University of Alberta
Printing Department

THE UNIVERSITY OF ALBERTA

THE LACTATE DEHYDROGENASES OF LYMPHOID AND OTHER ORGANS OF THE CHICKEN

by

GILBERT ALLAN SCHULTZ

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

SEPTEMBER, 1966

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Lactate Dehydrogenases of Lymphoid and Other Organs of the Chicken", submitted by Gilbert Allan Schultz in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Many important enzymes occur in multiple forms known commonly as isoenzymes. Lactate dehydrogenase (LDH) occurs in at least six forms in the chicken. We have detected only three of these in the bursa of Fabricius and thymus. These forms belong to the anodal group of LDH's which is the first group to appear during development. The LDH pattern of the bursa and the thymus does not change significantly during development and resembles that of brain and contrasts with the patterns of post-embryonic spleen, liver, kidney, and muscle which possess appreciable amounts of cathodal LDH. In fact, in gastrocnemius, there is a shift to almost pure LDH-5, the most cathodal isoenzyme. The absence or near absence of cathodal LDH from the primary lymphoid tissues would seem to account for the low LDH activity of these tissues.

Destruction and inhibition do not account for the absence of cathodal LDH in the bursa and thymus, nor is rapid secretion of cathodal LDH a likely explanation of its low concentration. Rather, it appears that the lymphocyte has either retained an embryological repression of cathodal LDH or has reacquired this repression.

The lymphoid organs resemble brain in their sensitivity to 19-nortestosterone. The marked drop in LDH activity levels after hormonal treatment in addition to their similar LDH isoenzyme patterns suggests that these organs may have some metabolic similarities.

ACKNOWLEDGMENTS

I acknowledge the support of Dr. R. F. Ruth throughout this entire project. In addition to guidance and advice in the direction of the research, his supervision of the writing of this thesis is greatly appreciated.

Laboratory space and some equipment and supplies were provided by the Departments of Chemistry and Zoology of the University of Alberta and additional equipment and supplies were made available through funds granted by the Medical Research Council of Canada. My own support during the entire duration of the study was provided by a Summer Supplement and Bursary from the National Research Council of Canada.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

> "According to the concept of the unity of biochemistry, all enzymes with the same function are created equal. However, some enzymes are created more equal than others."
>
> -- after George Orwell

The reducing power of tissues was first noted by Ehrlich in 1885. In 1910, Thunberg described the enzymatic oxidation of lactic acid and, in 1922, Wieland showed that biological oxidations can occur in the absence of oxygen and that the great majority of oxidations are dehydrogenations (Elvehjem, 1949). For 30 years thereafter, the lactate dehydrogenase (LDH) activity of each biological species was attributed to a single species of protein molecule. During this period heterogeneous preparations of aldolase, chymotrypsin, lysozyme, pepsin, trypsin, and xanthine dehydrogenase were described, but the separation of (ox heart) LDH into two electrophoretically distinct and active components was first described by Nielands in 1952. These two enzymes were very similar excepting a difference in solubility. In 1953, Krebs reported the separation of another dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, into four electrophoretically distinct and active components. Again, these components were very similar excepting a difference in solubility.

In 1957, Wieland and Pfleiderer described the electrophoretic separation of LDH into five active components and identified the most anodal as the characteristic LDH of pig

heart and the most cathodal as the characteristic LDH of pig liver. In 1959, Nisselbaum and Bodansky showed that the characteristic anodal LDH of heart and the characteristic cathodal LDH of liver are immunologically discrete, which means that they possess distinctive secondary or tertiary structure, or both. This immunochemical distinction was extended to include human LDH (Nisselbaum and Bodansky, 1961). In 1960, Plagemann, Gregory, and Wróblewski published two papers which laid the foundation for the future: (1) each LDH can be isolated by zone electrophoresis and recovered for quantitative analysis, (2) each LDH reacts optimally with pyruvate at a concentration of pyruvate peculiar to the LDH, (3) each LDH can be inhibited by specific antibodies, (4) each LDH reacts optimally with sulfite at a concentration of sulfite peculiar to the LDH, (5) the five principal LDH enzymes differ in all these respects by uniform, comparable increments, and (6) all five kinds of enzyme molecules are the same size, as first indicated by Neilands (1952), which excludes the possibility that they represent a series of monomeric to pentameric complexes or polymers.

Following the publication of this incisive set of exclusions, Appella and Markert (1961), and Markert (1962), suggested that the pentaploid character of LDH is due to the aggregation of two distinct polypeptides, A and B, to form all five of the possible tetramers (AAAA, AAAB, AABB, ABBB, BBBB). This idea was derived from the established structure of hemoglobin (Markert, 1963). These authors dissociated LDH into four units,

each of which is one-fourth the size of the LDH molecule. However, the first proof of the dual aggregation of distinct LDH polypeptides was obtained through a combination of two techniques, zone electrophoresis in gels (Smithies, 1955) and dehydrogenase cytochemistry.

Dehydrogenase cytochemistry takes its origin from Lakon's 1939 correlation of reducing activity with seed viability. In 1944, Jerchel and Möhle reported the redox potentials of tetrazolium salts, which are reduced by living cells. These potentials are equivalent to those of the nicotinamide-adenine dinucleotide (NAD) dehydrogenases. In 1947, Mattson, Jensen, and Dutcher demonstrated the dependence of tetrazolium reduction on NAD and a dehydrogenase. The complexity of this reaction is indicated in Shelton and Schneider's 1952 report of the need for an additional enzyme to mediate it. The details of the reaction were described in 1956 by Farber, Sternberg, and Dunlap. These authors also introduced phenazine methosulfate as a substitute for diaphorase, the mediate enzyme. In the same year, Tsou, et al. described the synthesis of the substituted tetrazolium salts which have become an invaluable part of cytochemical techniques. In 1959, Markert and Møller applied the cytochemical techniques for LDH to tissue extracts which had been subjected to electrophoresis in starch gels according to the technique of Smithies (1955). This led to the identification of seven LDH enzymes in tissues from one animal, serving to confirm the earlier report (Wieland and Pfleiderer, 1957) that one tissue may contain five or more electrophoretically distinct LDH enzymes. The sensitivity of

the technique perfected by Møller permitted the testing of many adult and fetal organs, and led to the discovery of shifts from one kind of LDH to another during development. The fact that these shifts are progressive, rather than abrupt, suggested that the changing patterns reflect gradual changes in the rates of synthesis of the A and B polypeptides which combine at random to produce the LDH patterns seen in electrophoretic gels.

Proof of the 'A and B' hypothesis, and strong support for the concept of random aggregation, was encountered following the accidental exposure of a mixture of the most anodal and the most cathodal LDH enzymes to a high concentration of salt (1M NaCl) instead of the low concentration which had been intended. The accident was discovered when the mixture was submitted to electrophoresis after it had been frozen, stored, and thawed in the solution of 1M NaCl. The mixture produced all five forms of LDH instead of the two expected. Markert (1963) interpreted this serendipitous observation to mean that a mixture of AAAA and BBBB had been dissociated by freezing in concentrated salt and had reaggregated at random to form AAAA, AAAB, AABB, ABBB, and BBBB. Indeed, the proportions of the different LDH enzymes conformed to expectation for random aggregation based on the binomial theorem, although strict proof of a binomial distribution was not advanced.

The LDH patterns of many vertebrate organs have been reported, but the lymphoid organs are prominent among the slighted. Our interest in the LDH patterns of the lymphoid organs is whetted by the unusual susceptibility of lymphocytes

to steroid hormones which decrease the utilization of glucose (Blecher, 1964). Moreover, the lymphoid organs undergo striking age-dependent changes; an early rapid growth and a later involution, both of which may be influenced by steroid hormones. No other vertebrate organs or tissues behave this way. Although steroid hormones would not be expected to activate or inactivate LDH, it is likely that an alteration of carbohydrate metabolism will entrain alterations in the levels and/or patterns of enzymes linked to carbohydrate utilization. In order to establish basic facts, we have compared the LDH activities of developing organs, including the lymphoid organs. We have also compared the LDH pattern of these organs and assessed the relative importance of each LDH. In addition, we have made preliminary observations of the androgen suppression of LDH activity in lymphoid tissue. We report a parallel between the LDH patterns of lymphoid tissue and central nervous tissue and similar susceptibilities of their LDH activities to androgen.

MATERIALS AND METHODS

"I love fools' experiments. I am always making them."

-- Charles Darwin

I. Terminology

The occurrence of multiple enzymes presents problems in nomenclature. Markert and Møller (1959) proposed the term 'isozyme' to describe different proteins occurring in one tissue or organ and having similar enzymic activity. However, the term 'isoenzyme' is to be preferred and is now officially recommended by the Standing Committee of Enzymes of the International Union of Biochemistry to describe multiple forms occurring in a single species. 'Isozyme' is an acceptable alternative if the individual author prefers it (Webb, 1964).

The numbering of isoenzymes is based on electrophoresis. Unfortunately, two contradictory systems have evolved. The one assigns number 1 to the isoenzyme with the greatest (anodic) mobility, and the other describes the slowest component as isoenzyme 1. The favored system is to number the isoenzymes in decreasing order of negative charge (Wilkinson, 1965). Thus, the most anodal LDH is LDH-1 and the others are numbered in succession.

Markert's experiments led to the hypothesis that two genes control the synthesis of five distinct, tetrameric isoenzymes distinguished from one another by their subunits. According

to the numbering system outlined above, Markert's terminology designates LDH-5 as AAAA, LDH-4 as AAAB, LDH-3 as AABB, LDH-2 as ABBB, and LDH-1 as BBBB.

Another nomenclature has been devised by Kaplan and co-workers. They designate the basic subunit for LDH-5 as muscle-type or 'M', and the basic subunit for LDH-1 as heart-type or 'H'. Then, according to the hypothesis that each isoenzyme is a tetramer composed of four subunits, the five isoenzymes are: LDH-5 = MMMM, LDH-4 = MMMH, LDH-3 = MMHH, LDH-2 = MHHH, and LDH-1 = HHHH. Therefore, Kaplan's 'M' is equal to Markert's 'A' and Kaplan's 'H' is equal to Markert's 'B'. Markert's terminology is used in this thesis although Kaplan's terminology will be used when reference is made to his work and that of his colleagues.

II. General Description of Chickens

All chickens used in this experiment were F_1 hybrids of two highly inbred lines maintained by Hy-line Poultry Farms, Johnston, Iowa. The birds are heterozygous for most of the blood group loci known in these strains of white leghorns. Eggs were incubated and hatched in a Jamesway Model 252 incubator at a constant temperature of 100 ± 0.2 F and 80% relative humidity. The eggs were turned automatically every six hours during the incubation period. Some embryos were taken at 11, 15, 17, and 19 days of incubation for use in the study. Others were transferred on the twentieth day to hatching trays and following hatching were removed from the incubator and placed in a Petersime Model 25D brooder for a period of four

to six weeks. During this period they had free access to both chick starter and water. Later they were transferred to developer cages. Post-hatching samples were taken at 1, 7, 14, 30, 60, and 120 days of age, and after 180 days, i.e. adult age. Equal numbers of each sex were sampled. All chicks were marked with wing tags as permanent identification.

III. Preparation of Samples

Brain, heart, kidney, leg muscle or gastrocnemius, liver, spleen, and the lymphoid organs, the thymus and the bursa of Fabricius, were removed from embryos and from chicks and chickens immediately after killing with ether. For embryos of 11, 15, 17, and 19 days of age, only heart, liver, leg muscle, and brain were fully analyzed. The other organs of eight embryos of each sex were pooled, but even then it was hard to get accurate data for kidney, spleen, thymus, and the bursa of Fabricius. For 1, 7, and 14 day chicks, organs of two chicks of the same sex were pooled.

The leg muscles of embryos and chicks cannot be cleanly dissected until the chick is more than two weeks old. Preparations from embryos and younger chicks are identified as 'leg muscle'. By one month of age, the gastrocnemius can be cleanly dissected and these preparations are identified as 'gastrocnemius'.

Once removed from the host embryo or chicken, organs were washed free of contaminating blood with cold water, weighed, and quick-frozen at -70°C and then stored at -20°C until the time of homogenization. When convenient (generally

about an hour after removal of the organs), the organs were thawed and homogenized in a simple homogenizer with a motor driven pestle (Tri-R Instruments, Jamaica 35, N. Y.). All homogenates were made in 1 ml of 0.6 M sucrose per 200 mg wet weight of tissue and spun at 10,000 rpm in a refrigerated centrifuge for 15 minutes. The supernatant containing the LDH activity was pipetted and used for electrophoresis and activity assays. Heart and gastrocnemius of older chickens were pulverized at -70°C, (Auto-pulverizer, Bio-chem Instruments Inc., 39 Decker St., Capiague, L. I., N. Y.) before homogenization. Repeat homogenization and centrifugation yielded negligible activity. All samples were analyzed within two days and were kept at 1°C until analyzed.

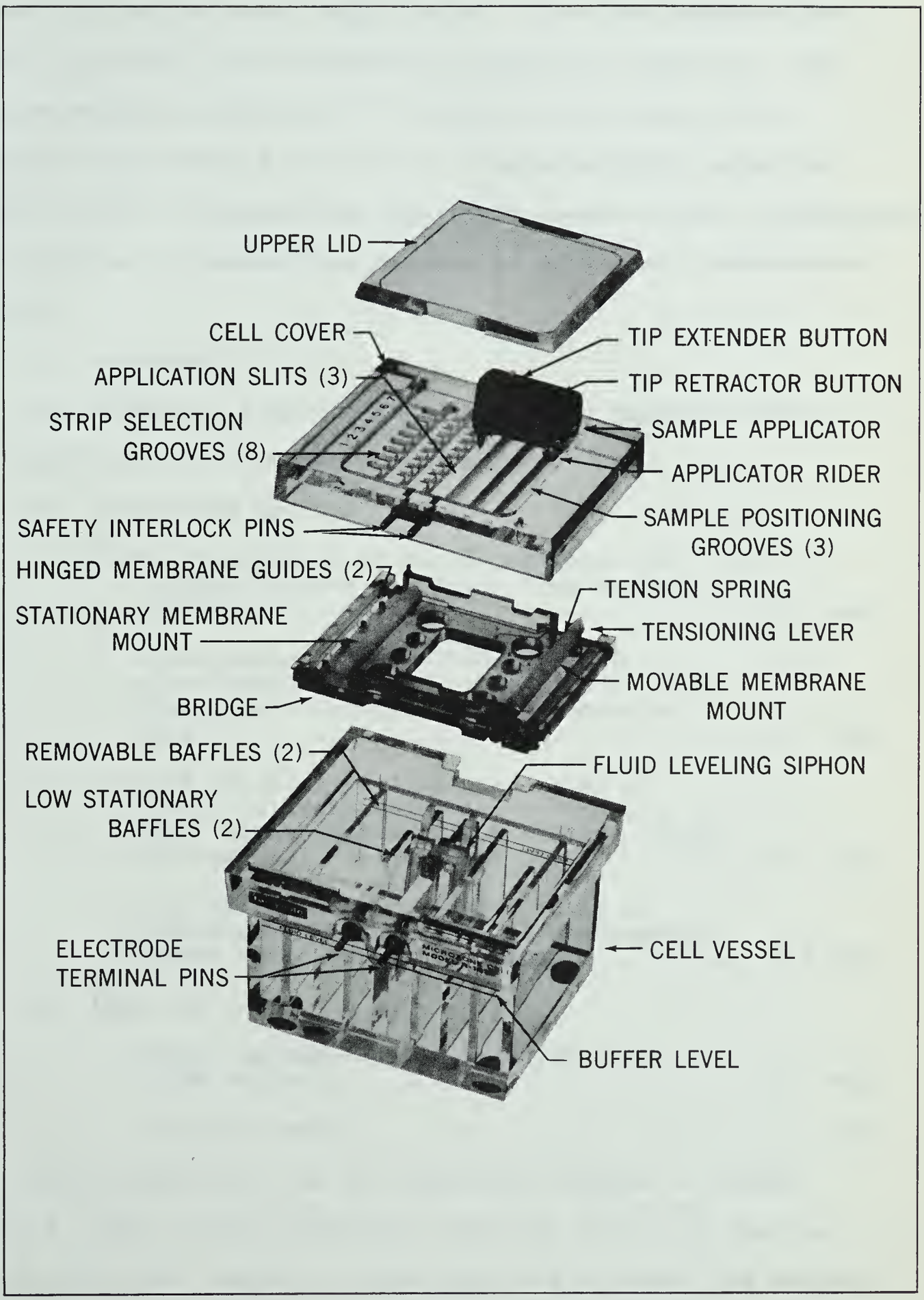
IV. Electrophoresis and Staining of LDH Isoenzymes

A. Electrophoretic separation

Electrophoresis was carried out in the Model R-101 Microzone Electrophoresis Cell (Beckman Instruments Inc., Stanford Industrial Park, Palo Alto, Cal.), which employs cellulose acetate membranes manufactured by Millipore Filter Corporation (Fig. 1). This membrane gives consistent migrations which is an important factor.

The membrane is prebuffered in 0.025 M Phosphate buffer, pH 6.2, by floating it on top of the buffer solution until saturated, immersing it for thirty seconds, and then blotting it lightly to remove excess buffer. In this way no air bubbles become trapped in the pores of the membrane. The membrane is then suspended in the bridge of the apparatus and placed in

Figure 1. 'Exploded' photograph of the Microzone Electrophoresis Cell (Beckman Technical Bulletin RB-TB-004).


UPPER LID
CELL COVER
APPLICATION SLITS (3)
STRIP SELECTION GROOVES (8)
SAFETY INTERLOCK PINS
HINGED MEMBRANE GUIDES (2)
STATIONARY MEMBRANE MOUNT
BRIDGE
REMOVABLE BAFFLES (2)
LOW STATIONARY BAFFLES (2)
ELECTRODE TERMINAL PINS
TIP EXTENDER BUTTON
TIP RETRACTOR BUTTON
SAMPLE APPLICATOR
APPLICATOR RIDER
SAMPLE POSITIONING GROOVES (3)
TENSION SPRING
TENSIONING LEVER
MOVABLE MEMBRANE MOUNT
FLUID LEVELING SIPHON
CELL VESSEL
BUFFER LEVEL

the electrophoretic cell filled with the same buffer and the connection to the power supply made. After the membrane has been in the cell for two minutes, samples are applied. The best separation requires 1-1/2 hours at 250 volts. The current varies from 2.5 to 6.0 milliamperes which warms the cell slightly. Separations exposed to normal room temperatures are superior to separations exposed to an ambient temperature of 1°C.

B. Staining

The method of staining is that used by Barnett (1964) and requires the following solutions:

1. incubation medium:

   - 0.1 M solution in distilled water of lithium lactate (British Drug Houses, London) ..... 1 vol.
   - 1% solution in distilled water of nicotinamide-adenine dinucleotide (NAD) (Cozymase - Nutritional Biochemicals Corp.) ..... 1 vol.
   - 0.1% solution in distilled water of 3(4,5 - dimethylthiazolyl - 2) - 2,5 - diphenyltetrazolium bromide (MTT) (Nutritional Biochemicals Corp.) ..... 3 vol.
   - 0.1% solution in distilled water of phenazine methosulfate (Dajac Reagents of the Borden Chemical Company) ..... 0.3 vol.

2. fixative

   - formal saline (4% formaldehyde in 0.85% saline) ..... 1 vol.
   - distilled water ..... 5 vol.

If the membrane with the separated enzymes is termed Strip A, then a second membrane (Strip B) is cut so that it is of sufficient length to cover the area in which the enzymes

have separated. Strip B is floated on the incubation medium in a petri dish so that it becomes fully saturated but not overloaded. This step is relatively critical because excess of incubation medium will cause diffusion of isoenzyme bands whereas insufficiency will cause premature drying during incubation. Strip B is then placed on a glass plate, avoiding air bubbles. This 'sandwich' is covered with Saran wrap to prevent evaporation and incubated at 37°C for 15 minutes. The strips are then fixed in formal saline for about 5 minutes and dried between filter paper to avoid curling. Bands are blue on a white or light blue background.

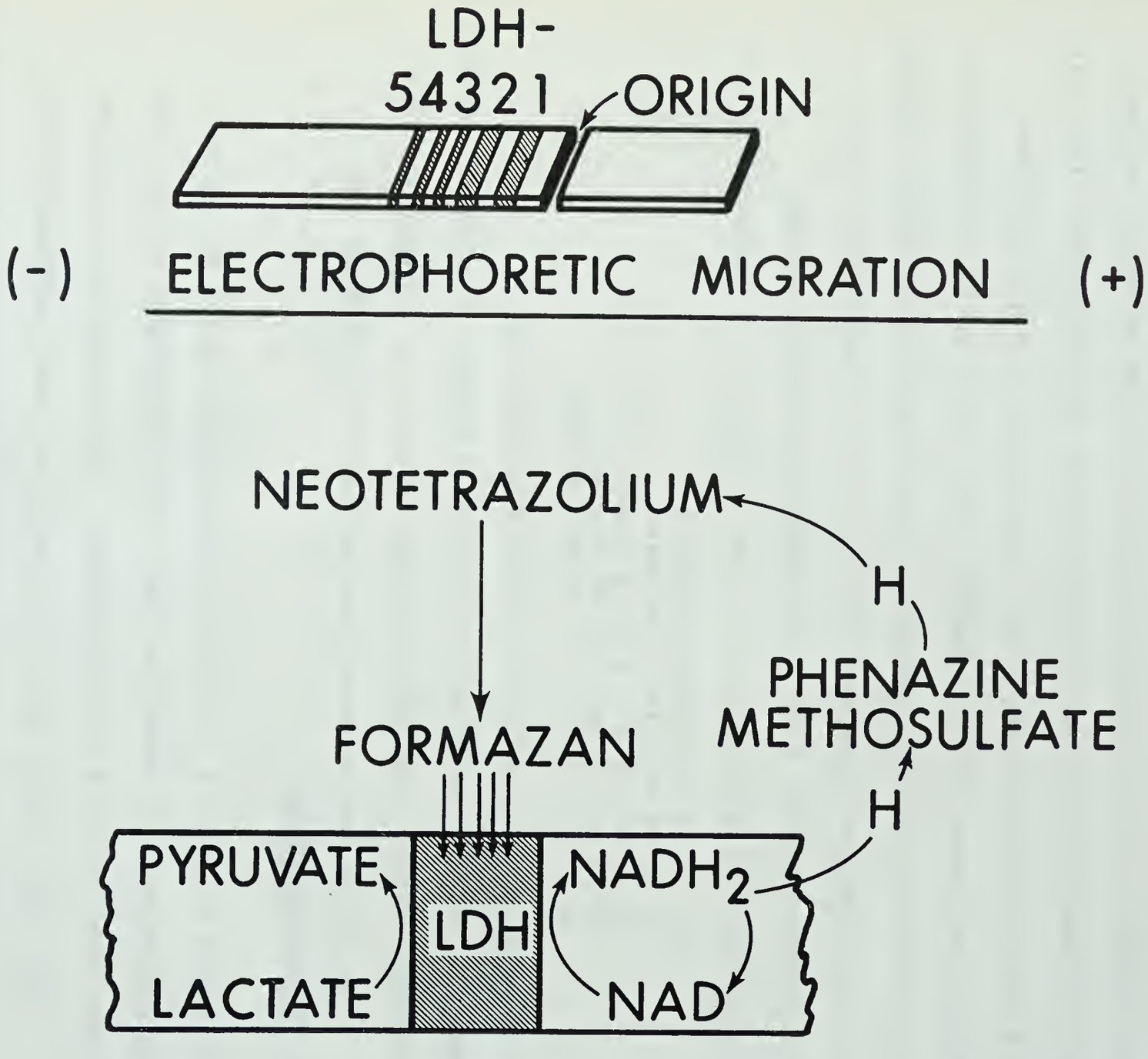
At pH 6.2, the isoenzymes all migrate toward the negative pole with LDH-1 the most anodal and LDH-5 the most cathodal isoenzyme. The staining involves the conversion of lactate to pyruvate with the concomitant production of $NADH_2$. The phenazine methosulfate catalyzes the transfer of hydrogen ion from $NADH_2$ to the soluble neotetrazolium salt (MTT) thereby converting it to the insoluble blue-colored formazan which precipitates at the site of enzyme activity as shown diagramatically in Fig. 2.

C. The pH

Chicken LDH did not migrate or segregate at the pH's routinely used for the electrophoretic separation of proteins. Hence, the following buffers were tried with chicken heart LDH, chicken liver LDH, and a commercial LDH (rabbit muscle LDH; Nutritional Biochemicals Corp.), as a standard:

Figure 2. (Upper) Diagramatic representation of the electrophoretic pattern of the LDH of chicken liver.

(Lower) The staining sequence used to demonstrate LDH activity. Hydrogen is transferred from lactate to $NADH_2$ to phenazine methosulfate to neotetrazolium which is irreversibly reduced to insoluble formazan. The sequence of reactions occurs rapidly and in the immediate vicinity of the LDH so that the precipitate of formazan marks the location of the enzyme. (modified from Markert and Ursprung, 1962).


LDH-
54321
ORIGIN
(-)
ELECTROPHORETIC MIGRATION
(+)
NEOTETRAZOLIUM
H
PHENAZINE
METHOSULFATE
FORMAZAN
H
PYRUVATE
LACTATE
LDH
NADH2
NAD

pH 10	0.1	M Tris buffer
pH 9.0	0.1	M Tris-HCl buffer
	0.03	M Borate - 0.025 M NaOH buffer
pH 8.6	0.07	M Barbital buffer
pH 8.0	0.025	M Phosphate buffer
pH 7.0	0.025	M Phosphate buffer
pH 6.2	0.025	M Phosphate buffer
pH 5.0	0.05	M Sodium acetate-acetic acid buffer

All runs lasted for 1-1/2 hours at room temperature, 250 volts, and 2.5 to 6.0 milliamperes. The difference between the migrations of mammalian and avian isoenzymes of LDH are portrayed in Fig. 3. The best separations occur at pH 6.2.

V. Activity Assays

Activity assays were done according to Markert and Ursprung (1962). One unit of LDH activity is defined as that amount of enzyme required to produce a change in optical density of 0.1 per minute at 340 mμ using a mixture of:

0.3	ml of 0.2 M lithium lactate
0.15	ml of NAD (10 mg per ml)
0.3	ml of 0.1 M Tris buffer, pH 9.0
2.25	ml of distilled water
10	μl of enzyme extract

Activities were measured in a 3 ml cuvette in a Shimadzu QV-50 Spectrophotometer: 1 cm light path; 340 mμ; 15 second intervals; ambient temperature, 22 $\pm$ 1°C. The initial rate of increase in optical density in the presence of excess lactate and NAD was generally the maximal rate as shown for an extract of 2 month chicken liver. In a few cases, initial rate was not the maximal rate, as in the 2 month chicken kidney (Fig. 4). The maximal rate of increase is used for the activity estimate and the units of LDH activity are calculated according to the following expression:

$$\text{Units LDH/ml} = \frac{\Delta \text{ O. D. per min X 3 X 100}}{0.1}$$

Figure 3. The electrophoretic behavior of rabbit muscle LDH (above) and chicken liver (L) and chicken heart (H) LDH (below). The drawing is a close copy of the relevant parts of several photographs (Room temperature, 1-1/2 hours, 250 volts, 2.5 to 6.0 ma).

ELECTROPHORETIC BEHAVIOR OF LDH vs. pH

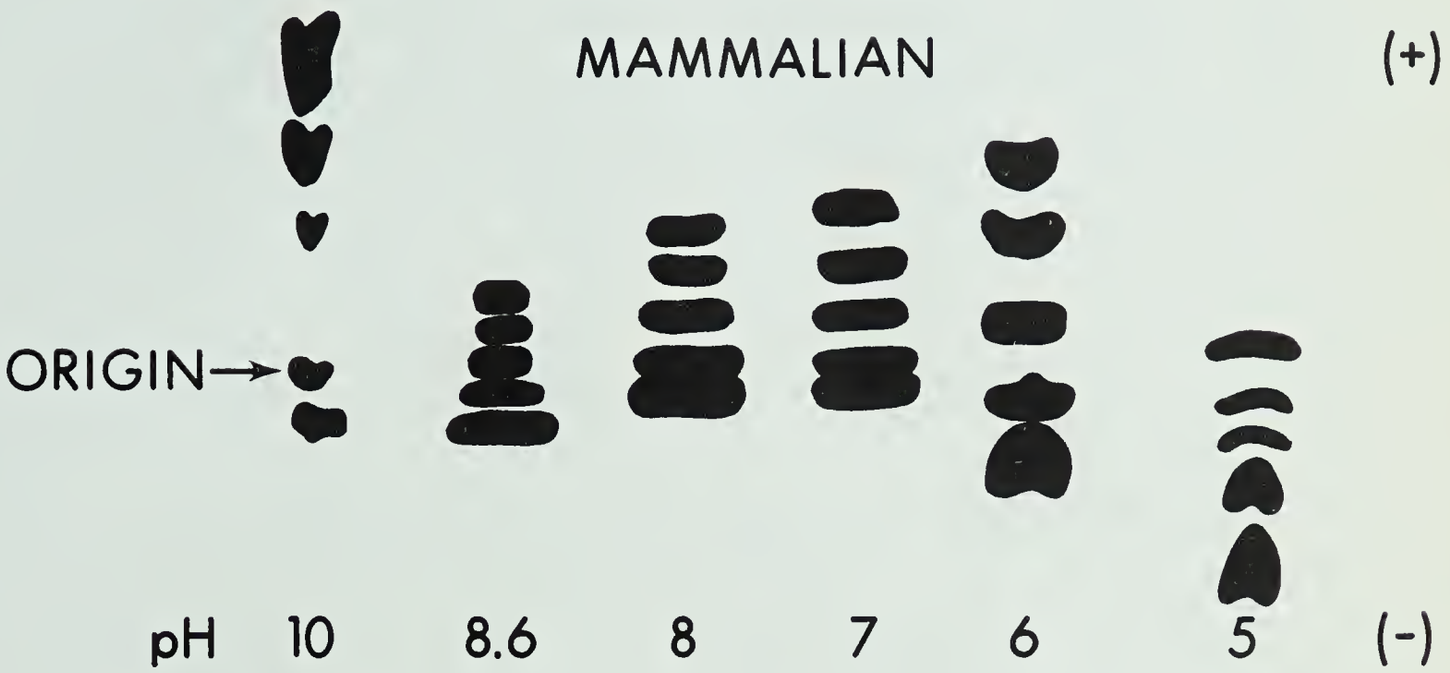
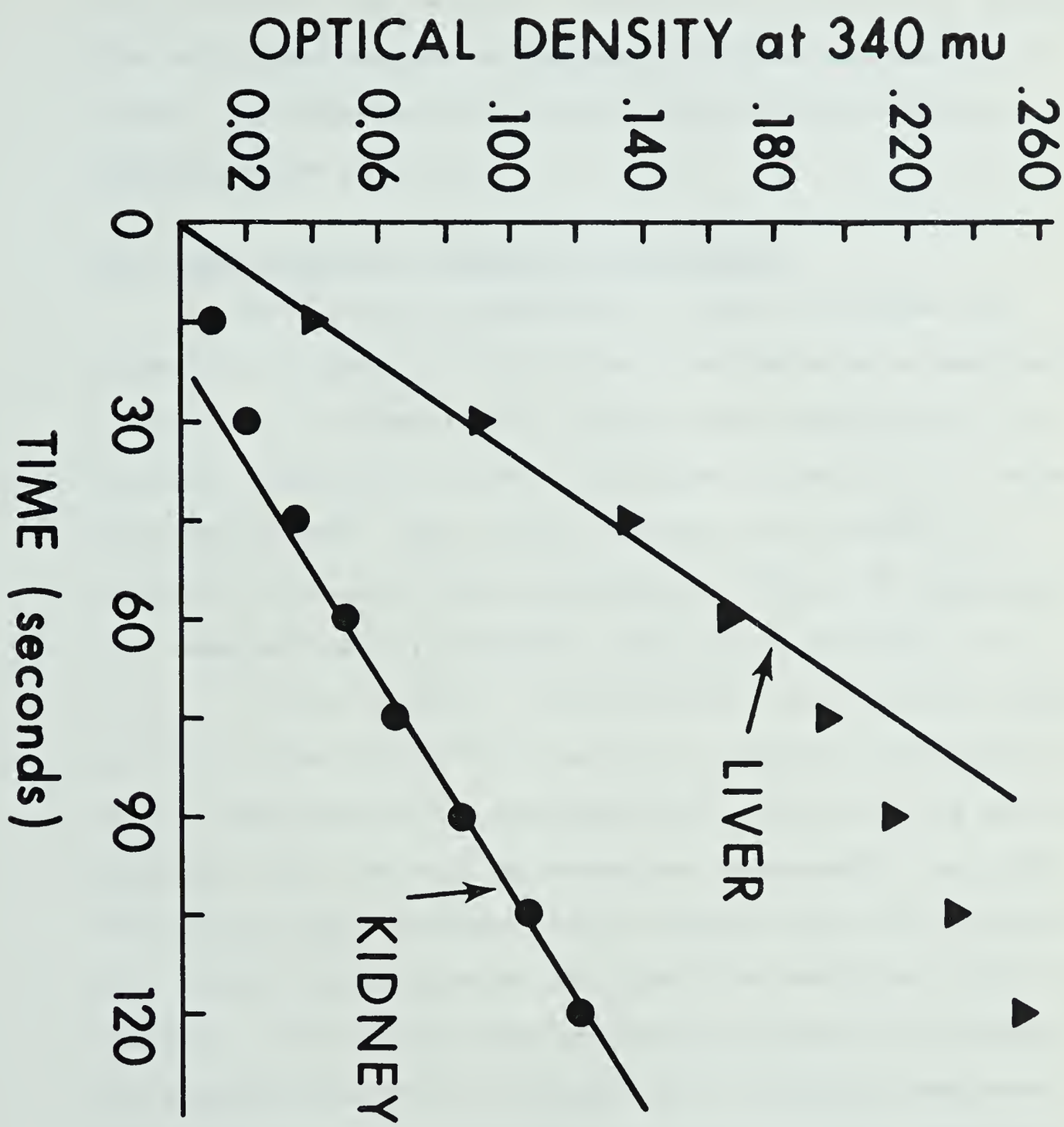


Figure 4. The changes in optical density due to the accumulation of $NADH_2$ as observed at 340 mμ in the presence of excess lactate and excess NAD. ▲ ; liver of a 2-month chicken. ● ; kidney of a 2-month chicken. The initial rate of the liver extract is also its greatest rate and this rate is estimated from the line fitted to initial points. The initial rate of the kidney extract is not its greatest rate so a line fitted to the greatest rate does not pass through the origin. The slope of the greatest rate per minute is divided by 0.1 to give the approximate units of LDH present in the extract. The molecular extinction coefficient of $NADH_2$ at 340 mμ is 6.22×10^6 sq $cm^{-1} \times mole^{-1}$ (Horecker and Kornberg, 1948).


OPTICAL DENSITY at 340 mu
0.02
0.06
.100
.140
.180
.220
.260
0
30
60
90
120
TIME (seconds)
LIVER
KIDNEY

OPTICAL DENSITY at 340 mμ
0.02
0.06
0.10
0.14
0.18
0.22
0.26
0
30
60
90
120
150
TIME (seconds)
ENZYME
ENZYME + ACTIVATOR

where 100 is the dilution factor and 3 is the number of millilitres in the cuvette. Since one millilitre of extract from any organ was the equivalent of 200 mg wet weight of tissue, the conversion to units/gram of tissue requires multiplication by five.

VI. Quantitation: Photovolt Densitometry

The LDH activity represented by each isoenzyme was estimated by scanning the stained electrophoresis membrane in a Model 52 - C Transmission Density Unit equipped with an automatic stage and a type C phototube connected to a Model 501-M Photometer. The optical density was recorded by a Model 42-B Varicord and integrated by a Model 49 Integrator (all manufactured by Photovolt Corp., 1115 Broadway, N. Y., N. Y.). A slit width of 1 mm was used. The Variable Response control of the Varicord was set at '1' which is identical with the 'L' position of the Densicord 542. This setting is not identical with the setting sometimes recommended for other uses of cellulose acetate strips scanned with the Densicord 542, perhaps because we do not clear the membranes prior to scanning. We obtained good agreement between the estimates of isoenzyme activities obtained with a Variable Response setting of '1' and independent estimates of the isoenzyme activities obtained by elution of sections of unstained strips.

Shredded sections of the cellulose acetate strips were eluted by high speed centrifugation. The first eluates contained 85 % to 90% of the total activity in concentrations adequate for rate determinations at 340 mμ. Table 1 records

the range of the estimates for each isoenzyme.

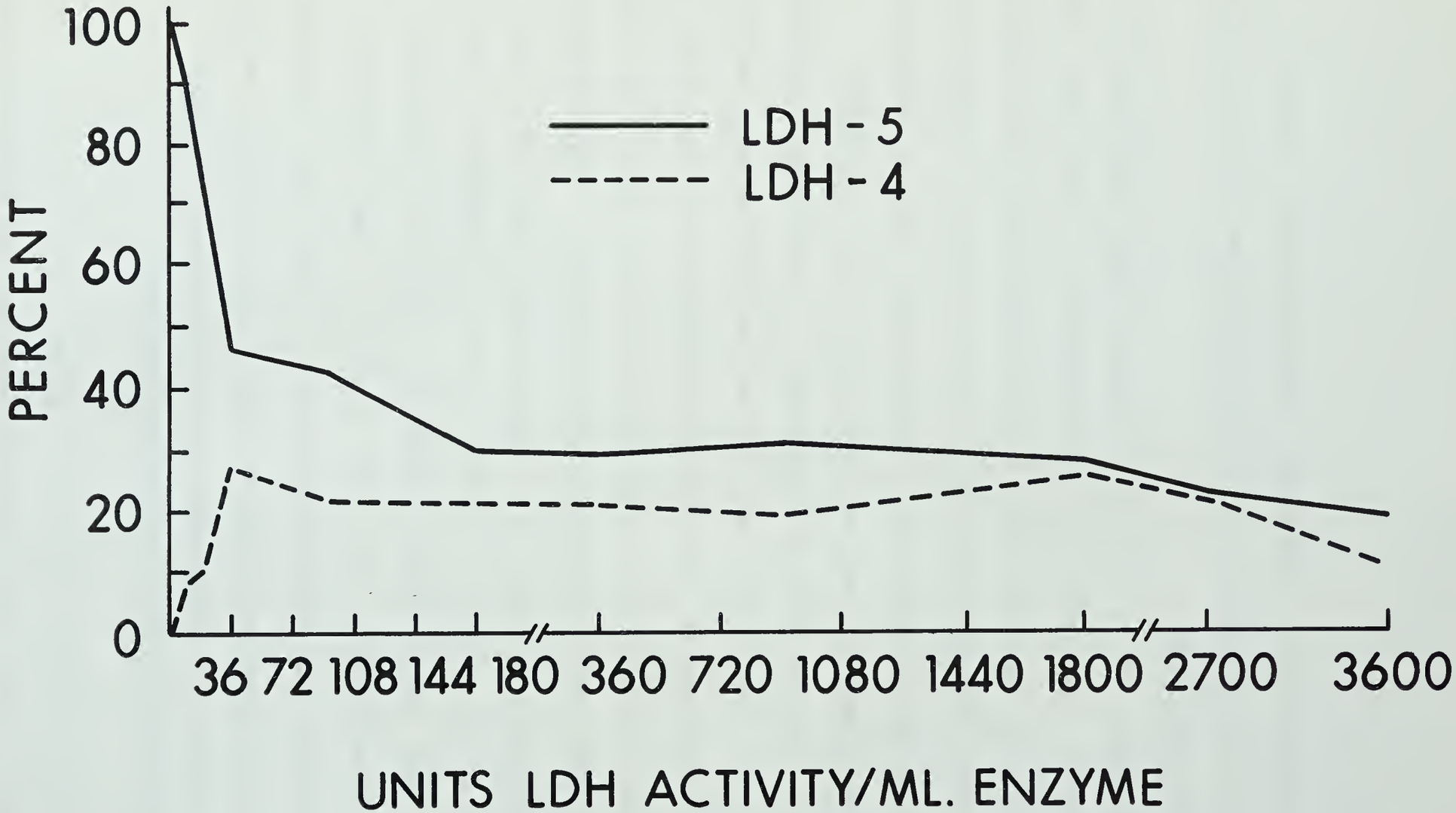
Table 1. A comparison of the assignment of percentile activities to isoenzymes of rabbit-muscle LDH by the elution method and by densitometry (Variable Response Control setting '1').

LDH isoenzyme	Elution method	Densitometry
1	9.9 - 13.3	8.2 - 12.8
2	9.8 - 11.1	6.8 - 10.8
3	11.5 - 15.2	10.7 - 20.8
4	18.6 - 22.3	17.1 - 20.7
5	43.2 - 45.9	39.2 - 49.1
Mean Total % Assigned	100.4%	98.1%

(All values are in percent and reflect the range of values obtained in four determinations for each method. The mean total % is the summation of assigned percentiles; it does not mean that 100% of the activity is recovered in the eluates.)

The optical densities of the membranes deviate from the straight line relationship given by Beer's Law. However, by setting the Variable Response Control at '1', a record closely approximating the proportional activities is obtained and tissue samples can be surveyed rapidly to infer the existence of quantitative differences among different tissues and bands, and to detect major deviations from expectation. Scans of a series of dilutions of rabbit muscle LDH (Nutritional Biochemicals Corp.) gave accurate estimates of the activities of LDH-4 and LDH-5 over a ten-fold range of activity: approximately 50 to 500 units/ml of each isoenzyme (Fig. 5). This range corresponds to a range of total activity, for all five isoenzymes, of 200 to 2000 units/ml.

Figure 5. The independence, from the effects of dilution, of the percentile activities assigned to LDH-4 and LDH-5. 3600 units/ml of rabbit skeletal muscle LDH was diluted up to 100 times. Each point in each curve represents the individual LDH isoenzyme activity as a percentage of the total LDH activity. Dilution has little effect on percentile assignments representing 50 to 500 units of LDH-5/ml and 20 to 600 units of LDH-4/ml. Most of our principal assignments of individual isoenzyme activities fall within these ranges.


100
80
60
40
20
0
PERCENT
LDH - 5
LDH - 4
36 72 108 144 180 360 720 1080 1440 1800 2700 3600
UNITS LDH ACTIVITY/ML. ENZYME

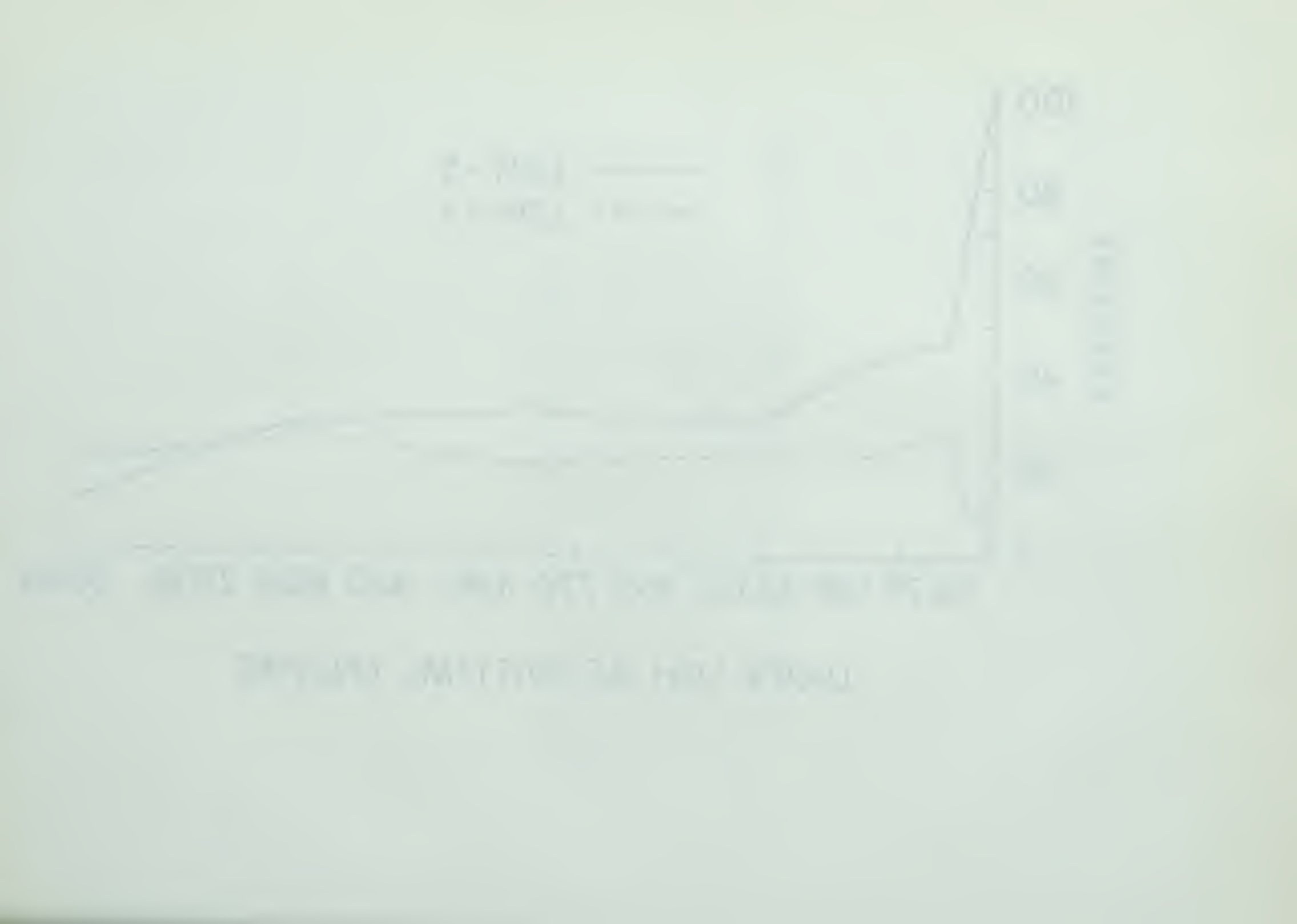


Figure 6 illustrates the scans obtained from chicken tissues. The area under each peak was calculated as a percentage of the total and then converted to activity units based on rate determinations at 340 mμ.

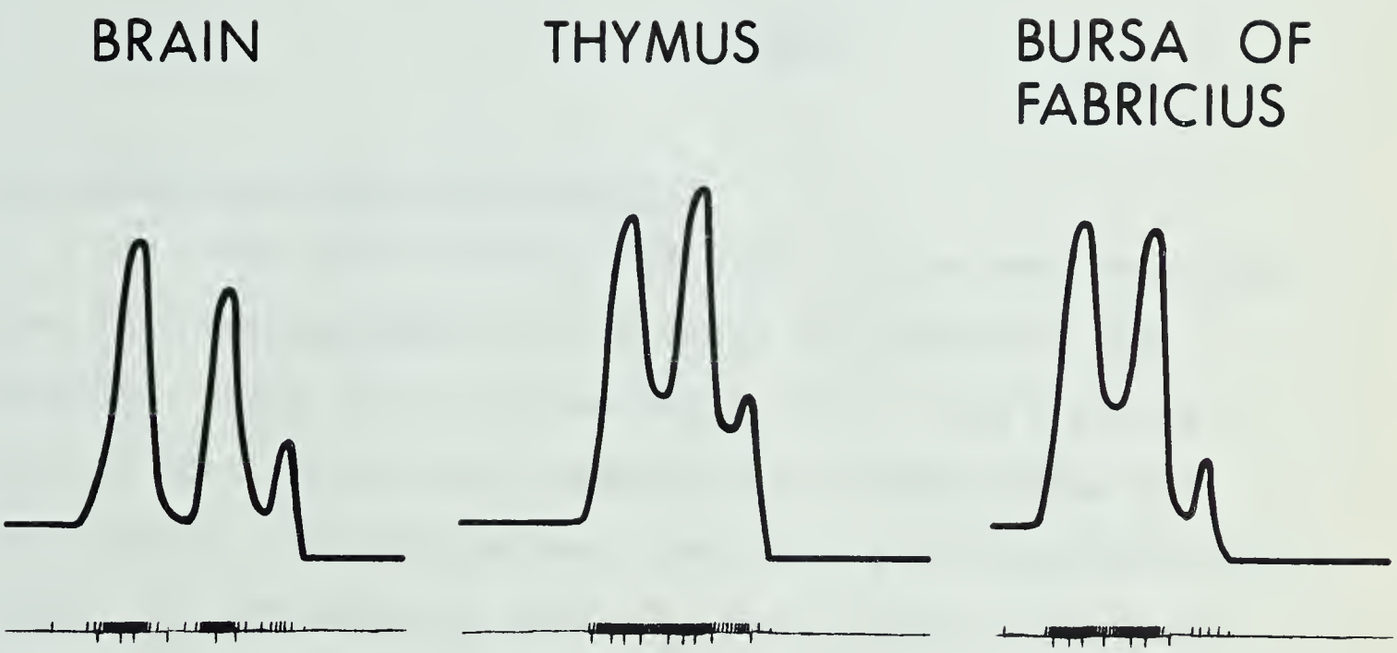
VII. Administration of 19-nortestosterone

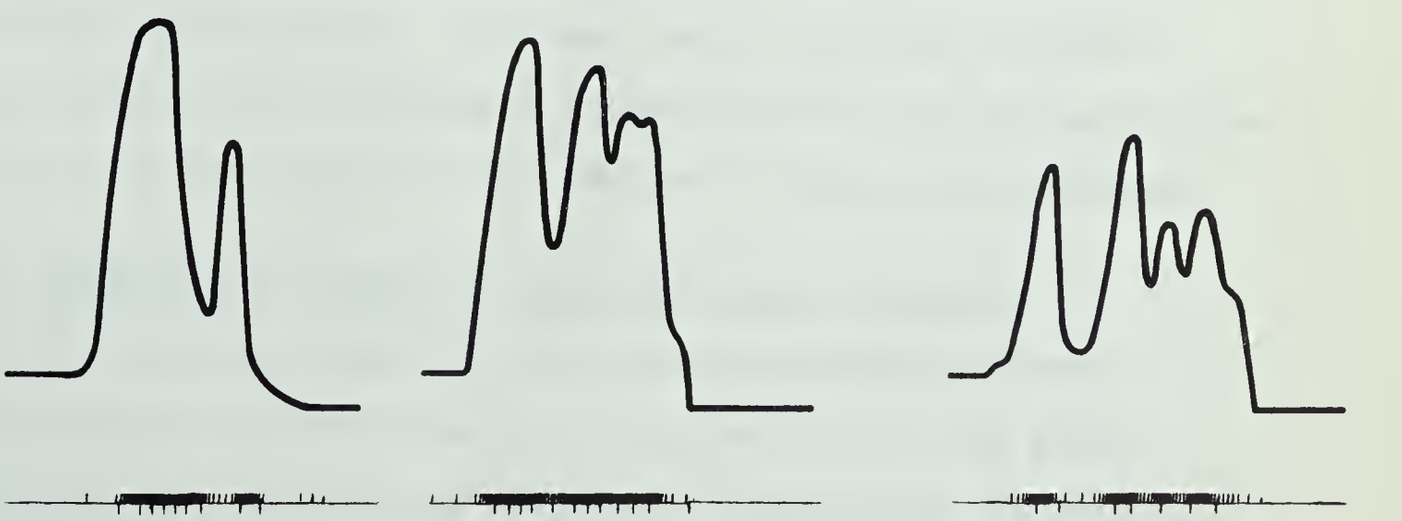
Two series of experiments were performed in which 19-nortestosterone was administered: first to chick embryos, and secondly to one-week old chicks. In the first, 0.63 mg of 19-nortestosterone in 0.1 ml of corn oil was injected directly into the albumen of eggs at 5 days of incubation according to the method of Meyer, Rao, and Aspinall (1959). Small holes were bored with a motor driven drill and after injection the hole was sealed with collodion. Sham controls using only corn oil for injections were also done. All eggs were then returned to the incubator (within one half hour) and brain, liver, heart, and leg muscle homogenates were made at 11, 15, 17, and 19 days of incubation. The study was extended to include thymus and spleen seven days after hatching.

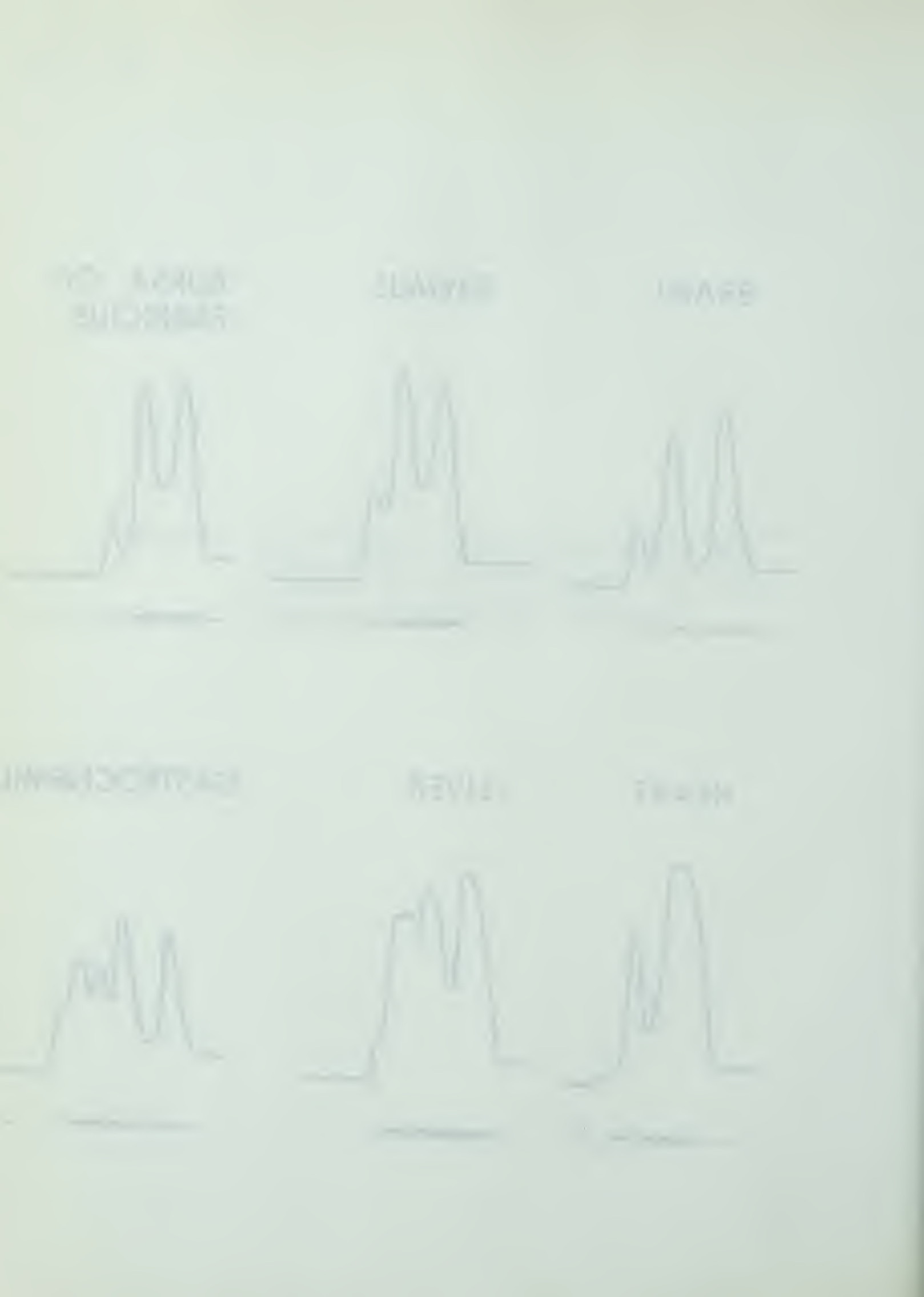
In the second experiment, chicks of 7 days of age received 1 mg of 19-nortestosterone in 0.1 ml of corn oil or corn oil alone, injected directly into the wall and cavity of the bursa of Fabricius. The injection was repeated the next day. Chicks were sacrificed at 10 and 12 days of age and homogenates of the bursas were made.

In both experiments, rate determinations, electrophoresis, and the estimation of isoenzyme activities were done as described.

Figure 6. Densitometric scans of the LDH activities of brain, thymus, bursa of Fabricius, heart, liver, and gastrocnemius of a 2-month old chicken. All scans represent a three-fold expansion of the actual migrations.


BRAIN
THYMUS
BURSA OF FABRICIUS


HEART
LIVER
GASTROCNEMIUS



RESULTS

"First get your facts; and then you
can distort them at your leisure."

-- Mark Twain

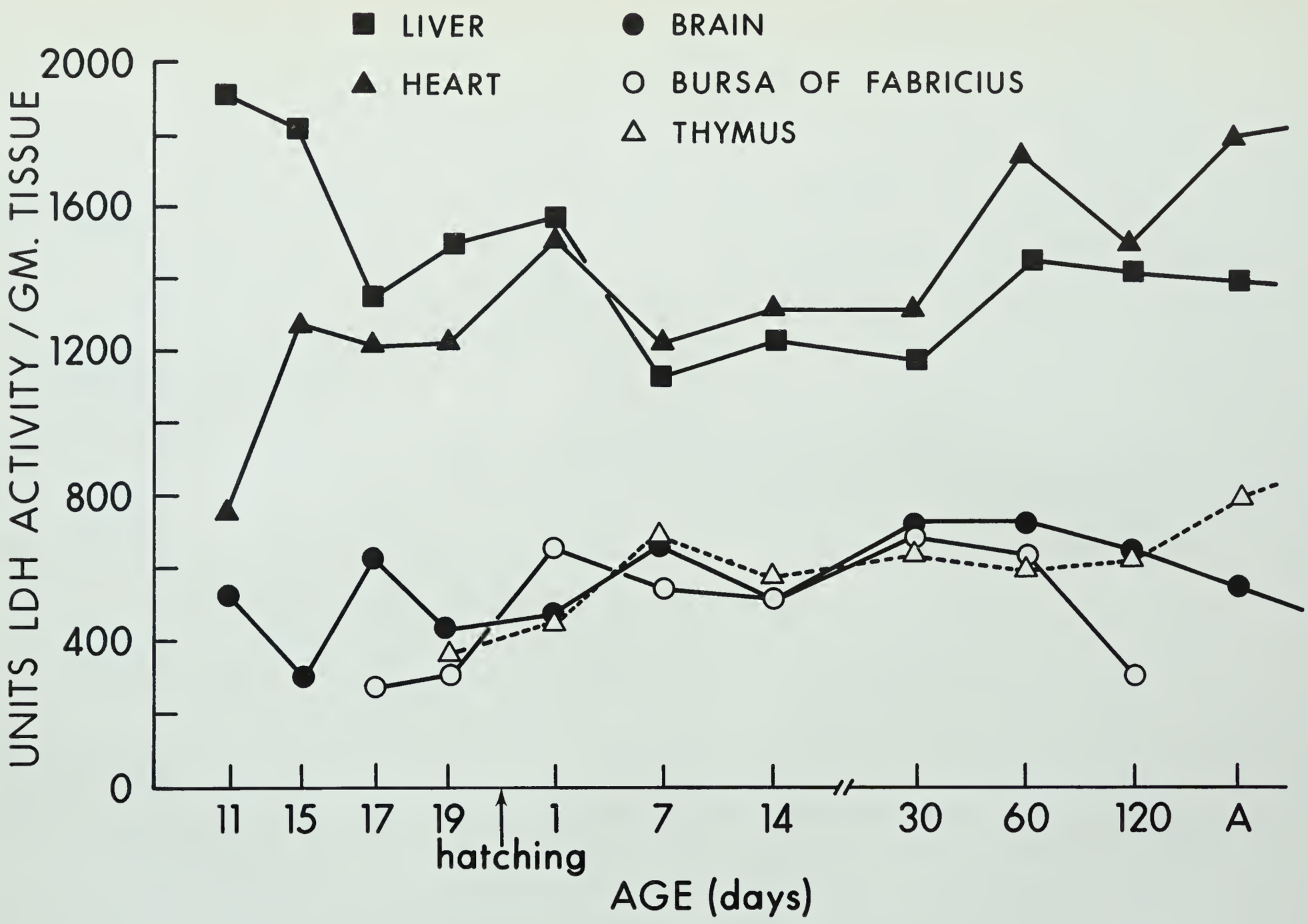
I. Changes in Total LDH Activity

The total LDH activities of eight organs were estimated from rate determinations as outlined in 'Materials and Methods'. Each value represented in Fig. 7 and 8 is the mean of four independent preparations for each stage of development. The graphs show that heart and liver have nearly double the LDH activity level of brain, thymus, and bursa (Fig. 7), while kidney, leg muscle/gastrocnemius, and spleen have intermediate levels of activity (Fig. 8). In addition to showing the general fluctuations in LDH activity through development, these means are basic to the conversion of the percentile activities of the isoenzymes to quantitative terms. Figures 7 and 8 include 76 means representing 304 independent estimations of the LDH activities of up to eight organs of 148 embryos, chicks, and chickens.

II. Qualitative Changes in LDH Isoenzyme Patterns

The extracts used for the rate determinations were analyzed electrophoretically. Figures 9 to 12 are photographs of the separations obtained at each stage of development. All separations were made in 1-1/2 hours with 0.025 M Phosphate buffer, pH 6.2, at 250 volts and 2.5 to 6.0 milliamperes. The membranes were stained by the method outlined

Figure 7. The LDH activity of liver, heart, brain, bursa of Fabricius, and thymus of the chicken. The thymus and bursa of young embryos are too small to test. One unit of LDH activity is defined as that amount of enzyme required to produce an increase in optical density of 0.1 per minute at 340 mμ in a mixture of excess lactate and NAD at room temperature. A = Adult.


LIVER
BRAIN
HEART
BURSA OF FABRICIUS
THYMUS
UNITS LDH ACTIVITY / GM. TISSUE
2000
1600
1200
800
400
0
11
15
17
19
hatching
1
7
14
30
60
120
A
AGE (days)

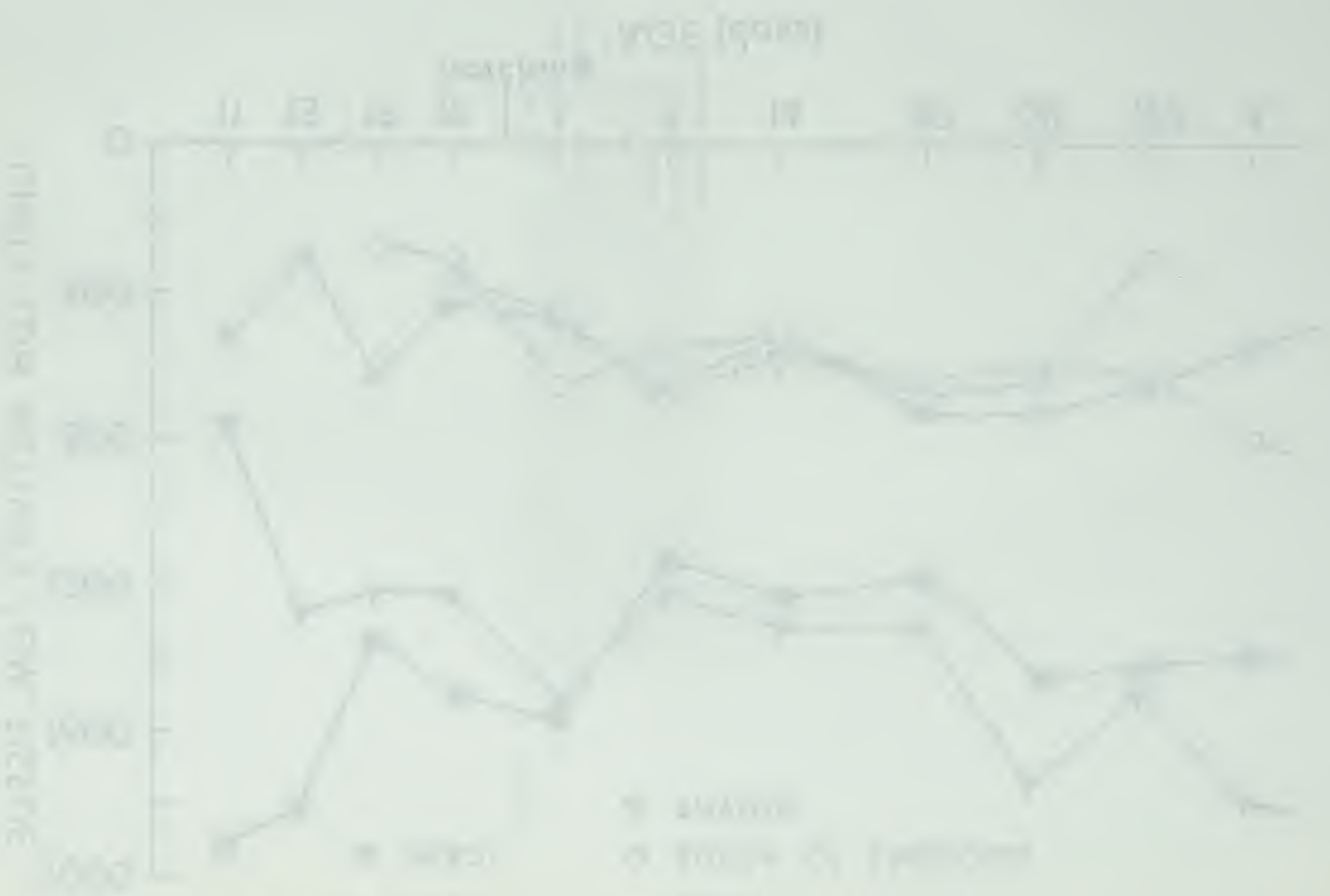
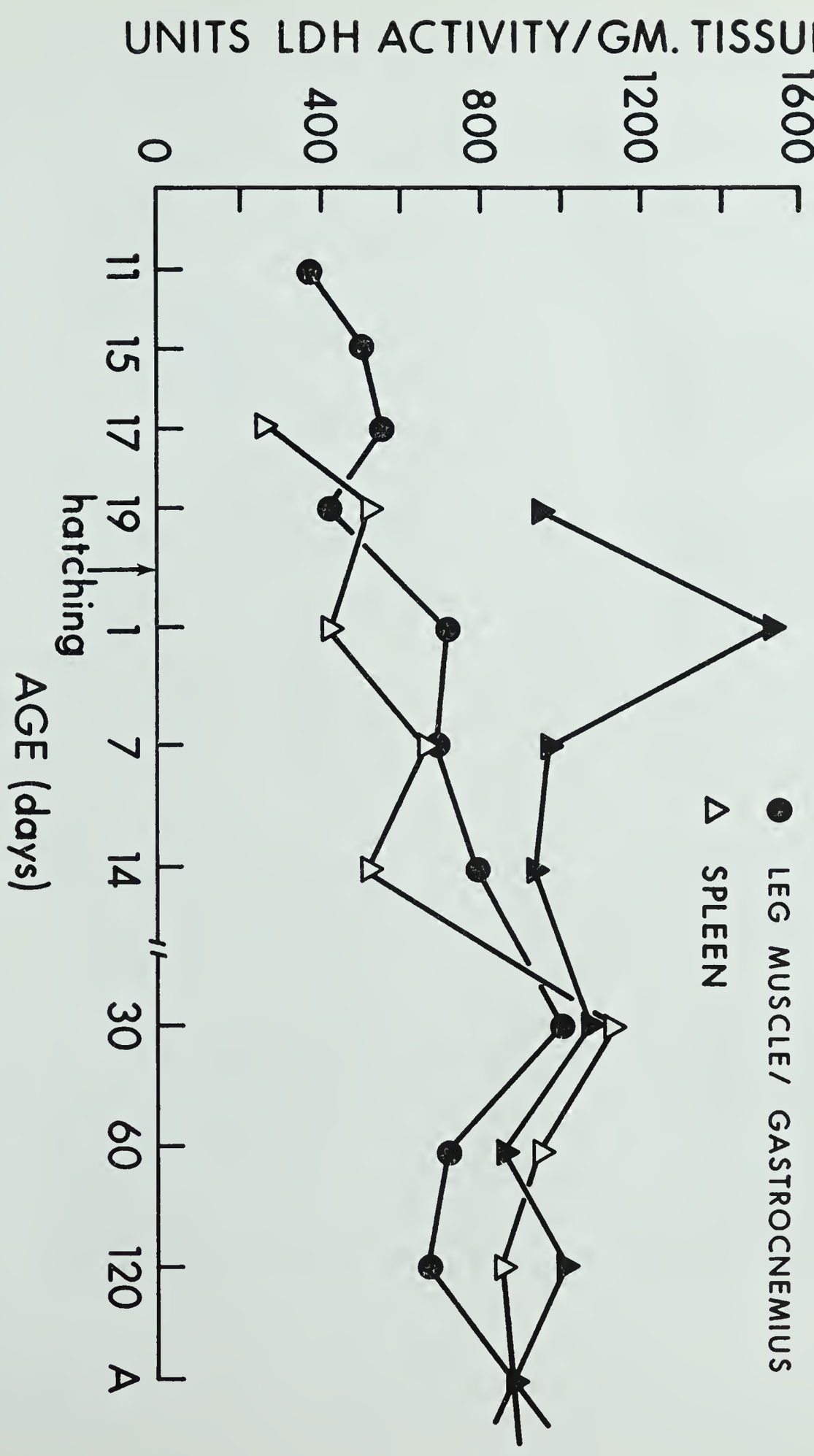
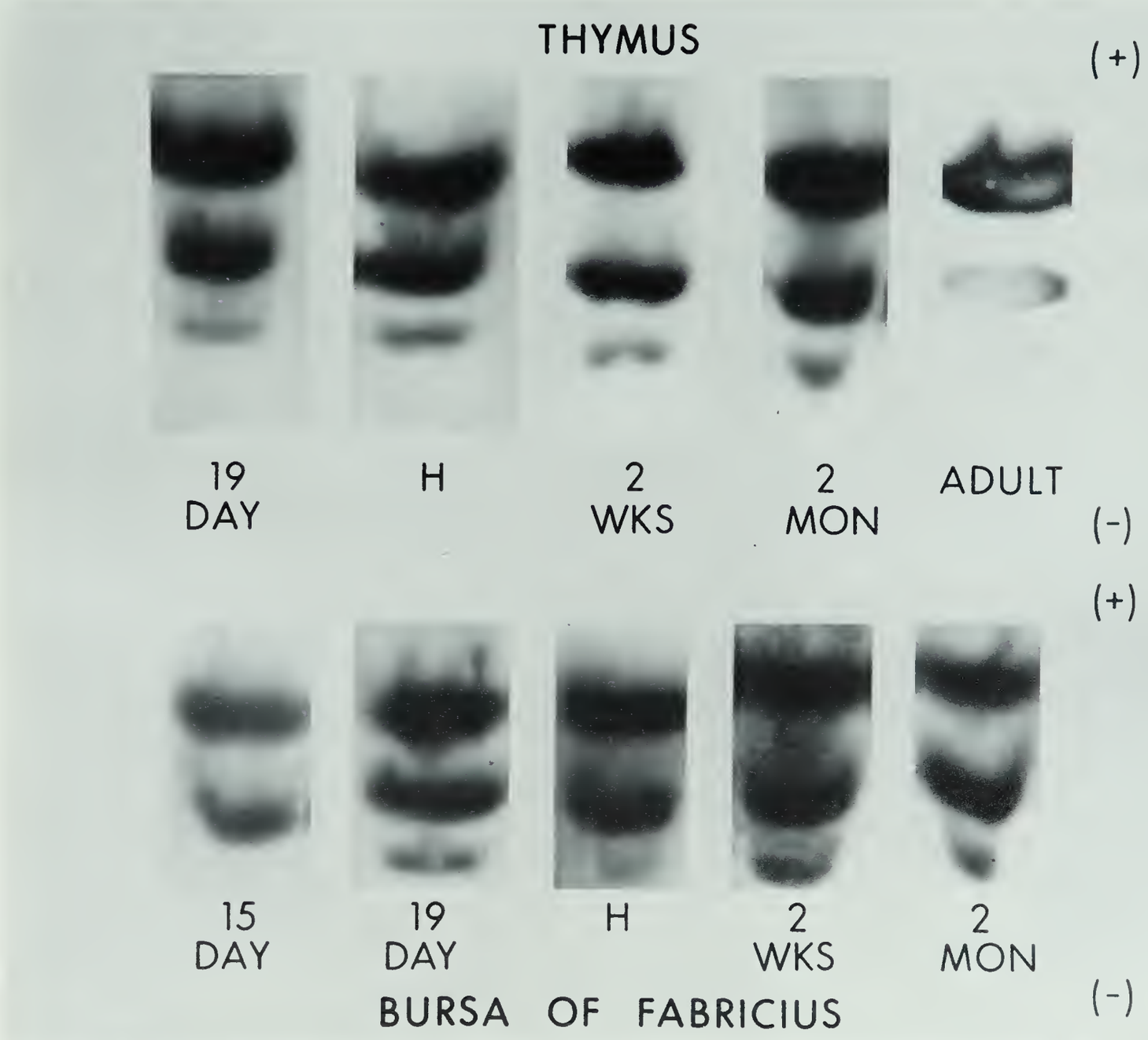
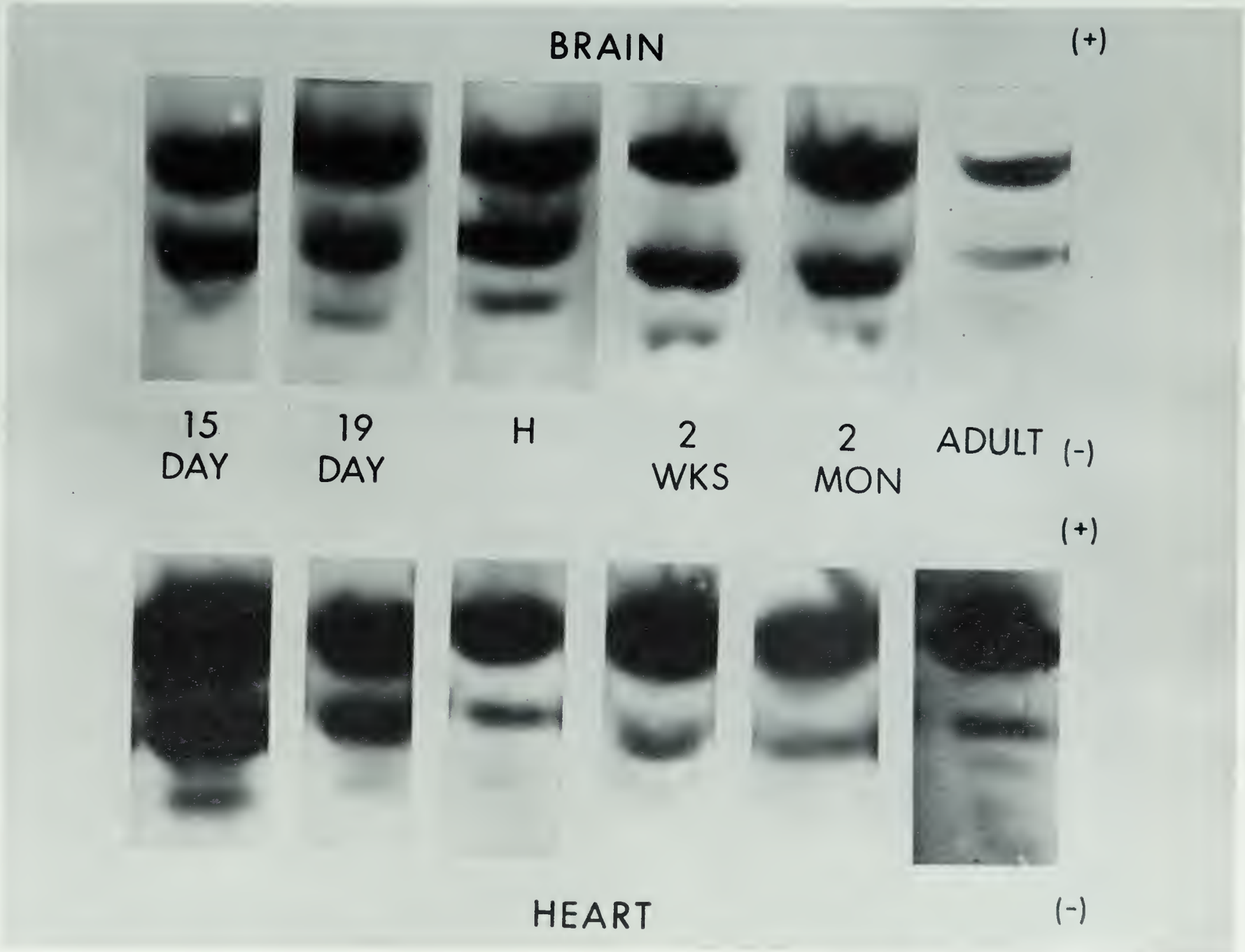


Figure 8. The LDH activity of spleen, leg muscle/ gastrocnemius, and kidney of the chicken. The spleen of young embryos is too small to test. Gastrocnemius is not exactly identified until 30 days of age. Units of activity and conditions are the same as described in Fig. 7. A = Adult.

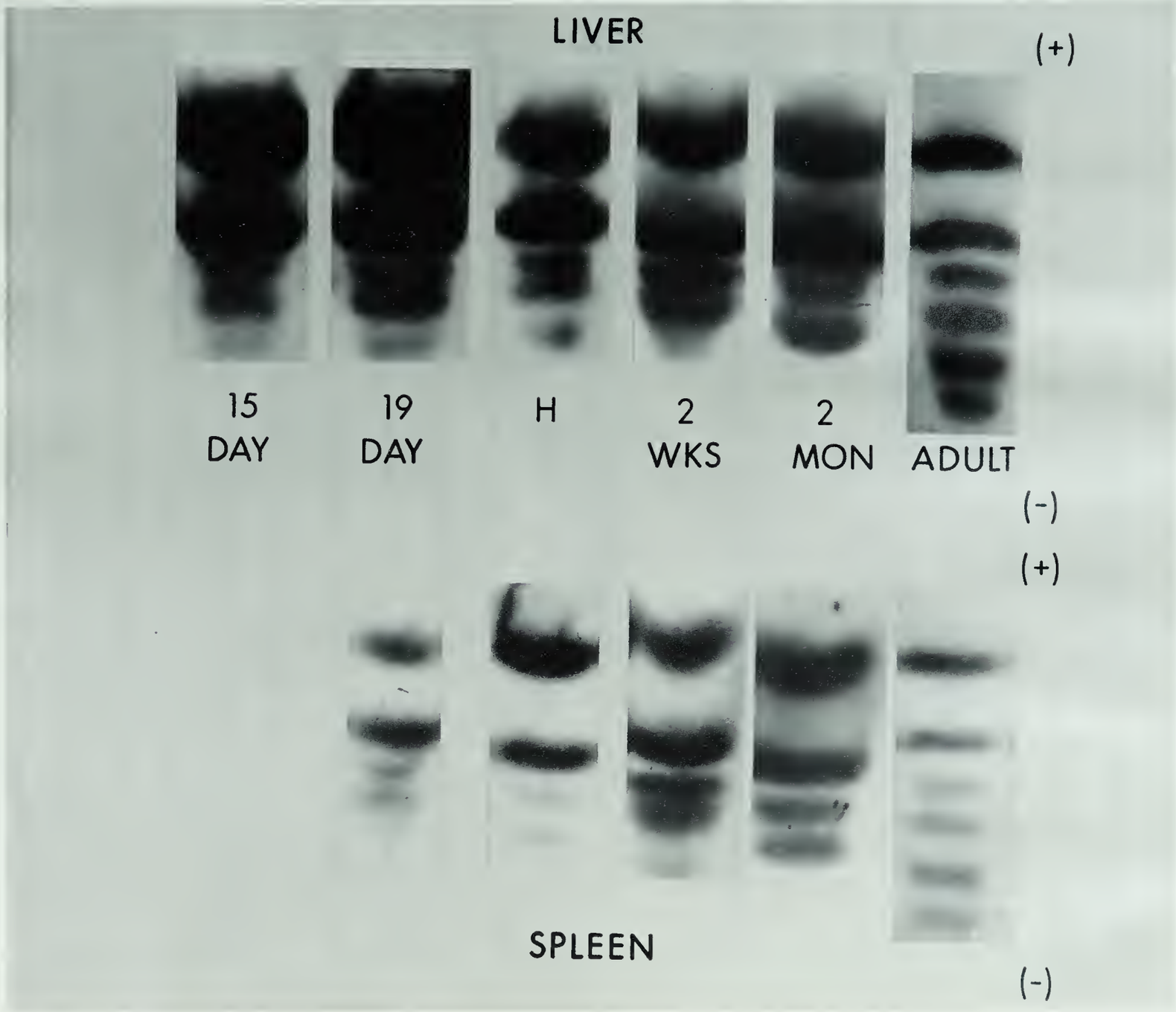

UNITS LDH ACTIVITY/GM. TISSUE
0
400
800
1200
1600
11
15
17
19
hatching
1
7
14
30
60
120
A
AGE (days)
KIDNEY
LEG MUSCLE/ GASTROCNEMIUS
SPLEEN

Figures 9, 10, 11, and 12. Photographs of electrophoretic patterns of LDH isoenzymes from several organs of the chicken during various stages of development. Electrophoresis was carried out on cellulose acetate membranes with 0.025 M Phosphate buffer, pH 6.2, at room temperature and 250 volts for 1-1/2 hours with a current of 2.5 to 6.0 milliamperes. Staining was as outlined in 'Materials and Methods'. 15 DAYS and 19 DAYS are days of incubation before hatching. H is one day post-hatching, 2 WKS is 2 weeks post-hatching age, and 2 MON is 2 months after hatching. The anode and cathode are designated by plus and minus while the origin lies anodal to LDH-1, the most anodal LDH isoenzyme. Under these conditions, six LDH isoenzymes were observed in the LDH patterns of 2 out of 4 adult livers and spleens sampled. Magnification X3.


THYMUS
(+)
19 DAY
H
2 WKS
2 MON
ADULT
(-)
(+)
15 DAY
19 DAY
H
2 WKS
2 MON
(-)
BURSA OF FABRICIUS


BRAIN
(+)
15 DAY
19 DAY
H
2 WKS
2 MON
ADULT
(-)
(+)
HEART
(-)


GASTROCNEMIUS
(+)
15 DAY
19 DAY
H
2 WKS
2 MON
ADULT
(-)
(+)
(-)
KIDNEY


LIVER
(+)
15 DAY
19 DAY
H
2 WKS
2 MON
ADULT
(-)
(+)
SPLEEN
(-)

previously. For details, see 'Materials and Methods'. The anode and cathode are designated by + and -. The origin is anodal to the top band of enzyme activity. These figures show that the electrophoretic segregation of chicken LDH can be reproduced systematically despite the slow migration and the belated separation, which are sluggish by comparison with mammalian LDH. The presence of an LDH-6, in some of the preparations, is ignored because its contribution to the total activity is trivial and its occurrence is sporadic. Other than noting it we have nothing worthwhile to say about it here.

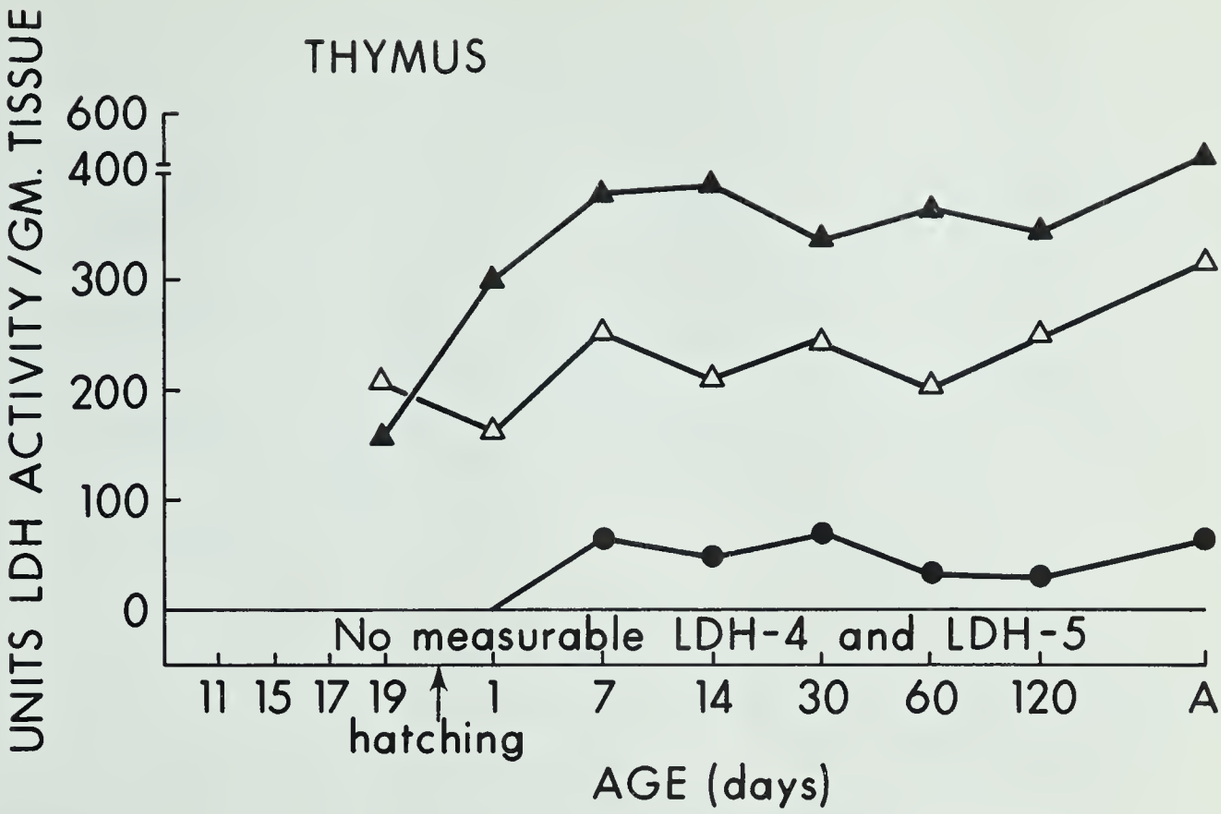
III. Quantitation of Isoenzyme Activities

Each stained electrophoresis membrane was scanned densitometrically and the percentile activity for each isoenzyme was obtained by integration. A mean percentile activity, for each isoenzyme of each organ at each stage of development, was obtained from the percentile activities of four independent preparations, each representing one to eight birds. The mean percentile activities were converted to quantitative terms with the aid of the mean total activities, obtained from the rate determinations of the four independent preparations. The activity of each isoenzyme of each organ at each stage of development is presented in Figs. 13 to 16. These figures show that there are real differences in the isoenzyme activities of the various organs. The activity of thymus LDH-1 (Fig. 13) tends to exceed the activity of thymus LDH-2, which is slightly less

Figure 13. The individual activities of the isoenzymes of the thymus and bursa of Fabricius during development and growth. Activities were obtained by converting percentages from densitometric to absolute terms using the values of Figs. 7 and 8. Units are as described in 'Materials and Methods'. Symbols are:

LDH-1 ▲
LDH-2 △
LDH-3 ♦
LDH-4 □
LDH-5 ■

A = Adult


THYMUS
UNITS LDH ACTIVITY/GM. TISSUE
600
400
300
200
100
0
No measurable LDH-4 and LDH-5
11 15 17 19 1 7 14 30 60 120 A
hatching
AGE (days)

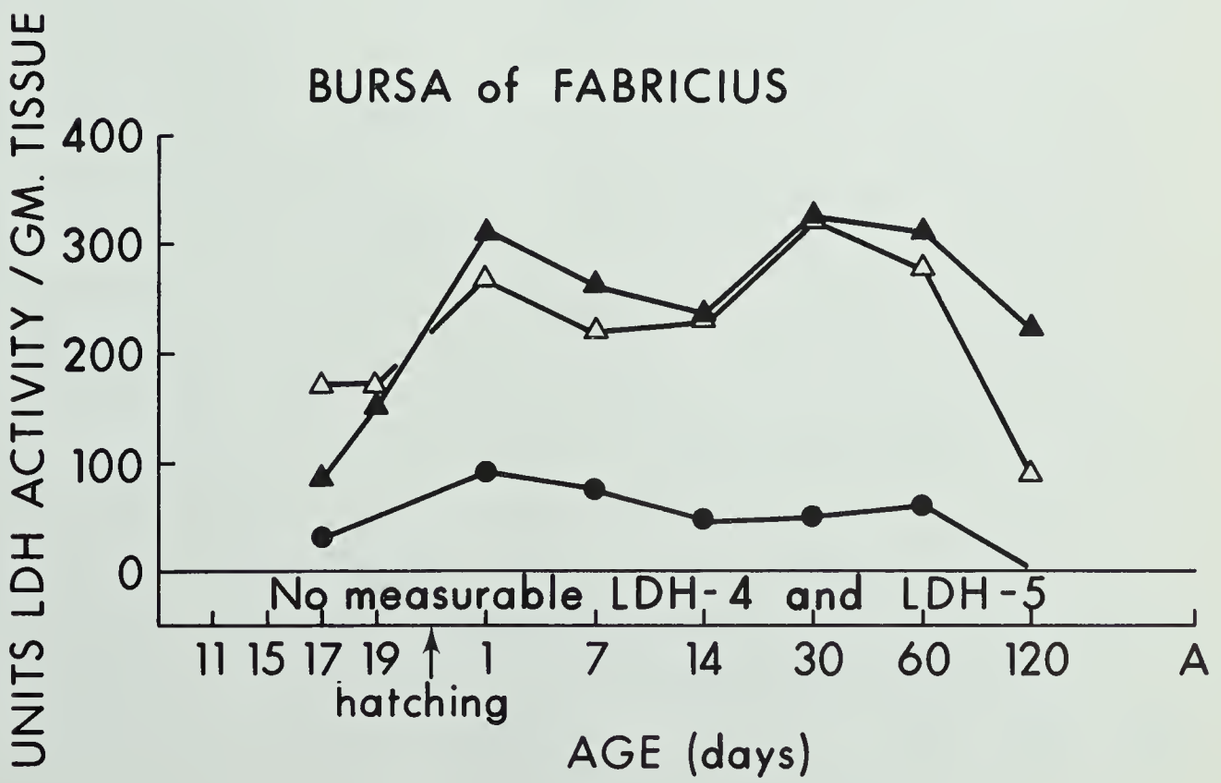
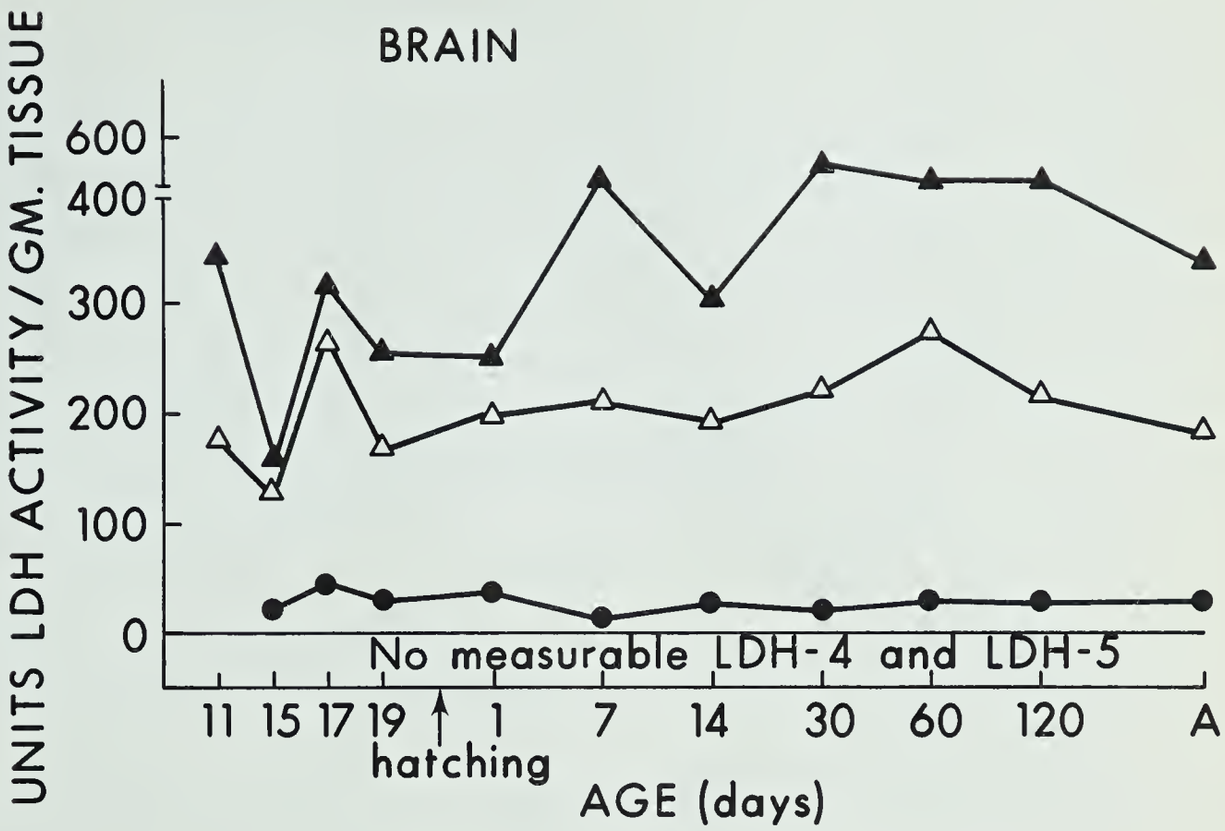

BURSA of FABRICIUS
UNITS LDH ACTIVITY /GM. TISSUE
400
300
200
100
0
No measurable LDH-4 and LDH-5
11 15 17 19 1 7 14 30 60 120 A
hatching
AGE (days)

Figure 14. The individual activities of the LDH isoenzymes of the brain and heart during development and growth. The symbols are the same as those given in Fig. 13 and the figure is directly comparable to Fig. 13.


BRAIN
UNITS LDH ACTIVITY/GM. TISSUE
600
400
300
200
100
0
No measurable LDH-4 and LDH-5
11 15 17 19 1 7 14 30 60 120 A
hatching
AGE (days)

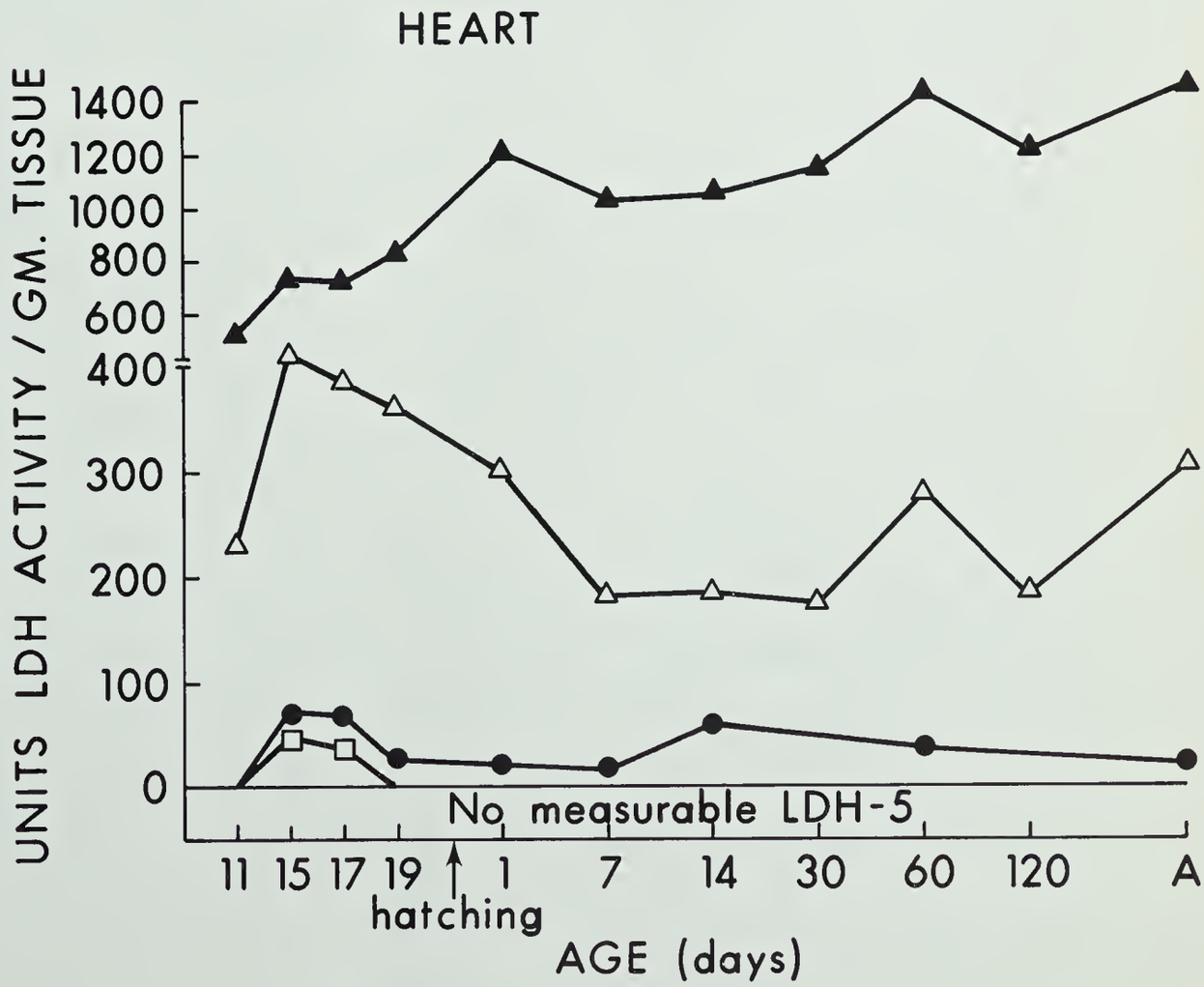
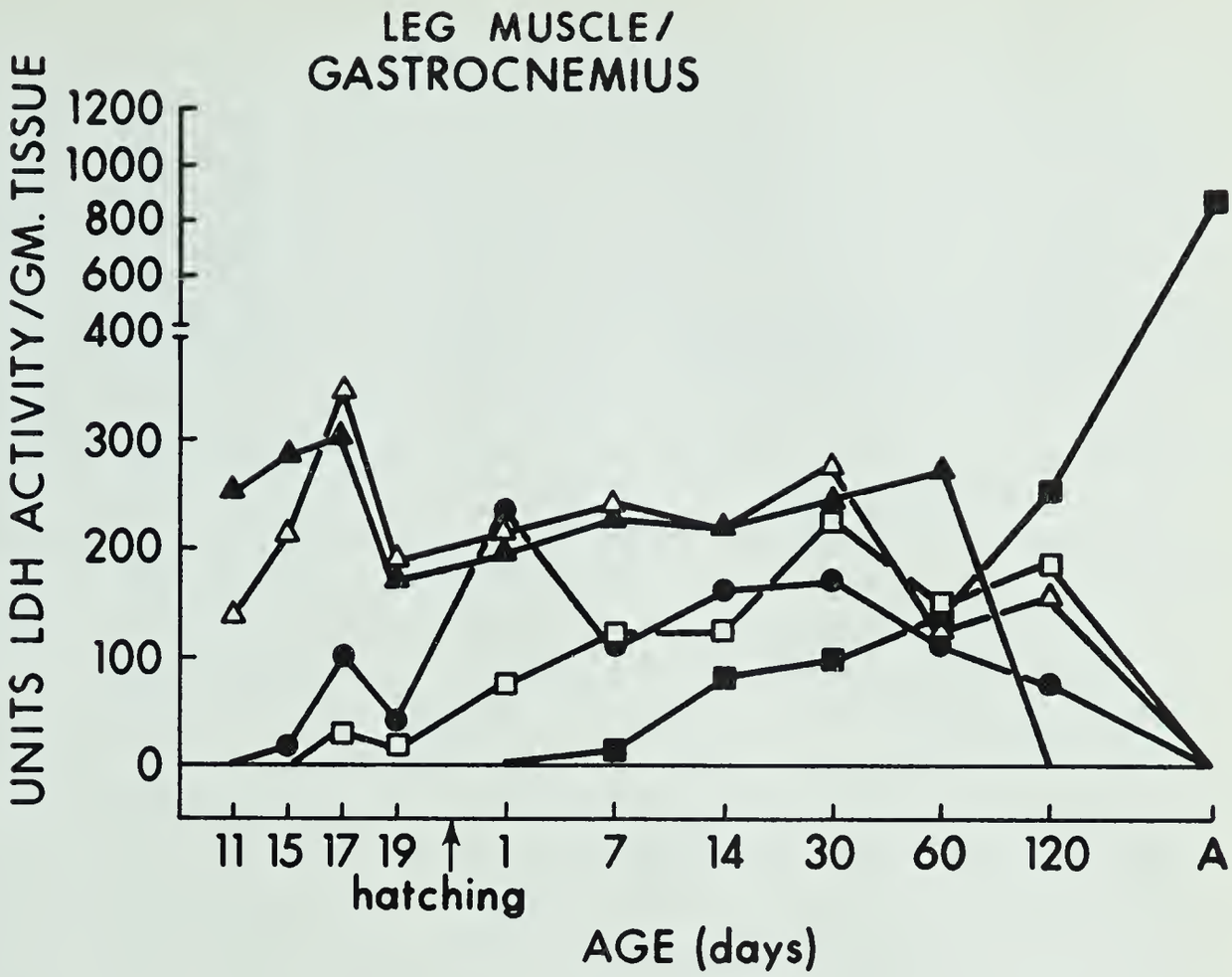

HEART
UNITS LDH ACTIVITY / GM. TISSUE
1400
1200
1000
800
600
400
300
200
100
0
No measurable LDH-5
11 15 17 19 1 7 14 30 60 120 A
hatching
AGE (days)

Figure 15. The individual activities of the LDH isoenzymes of leg muscle/gastrocnemius and of kidney during development and growth. The symbols are the same as given in Fig. 13. The figure is directly comparable to Fig. 13.


LEG MUSCLE/
GASTROCNEMIUS
UNITS LDH ACTIVITY/GM. TISSUE
1200
1000
800
600
400
300
200
100
0
11 15 17 19 1 7 14 30 60 120 A
hatching
AGE (days)

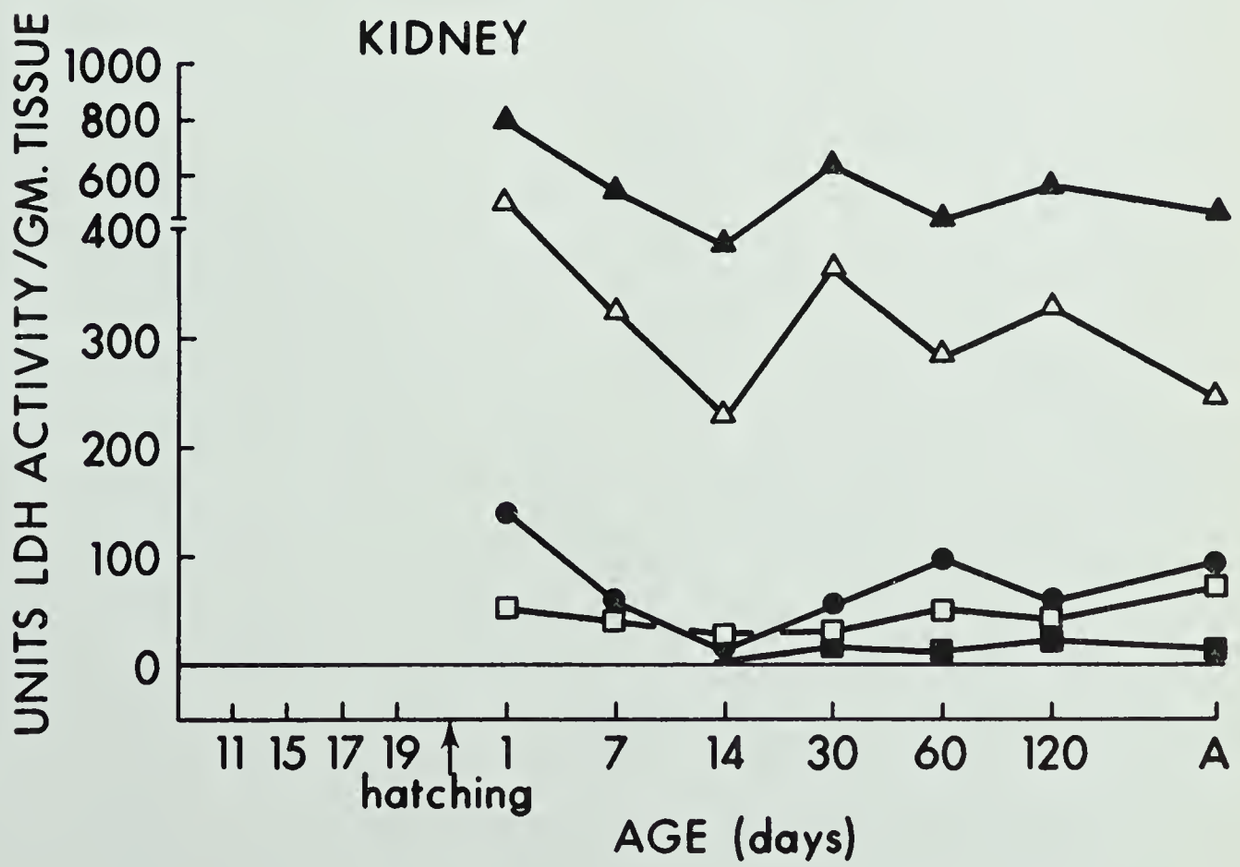
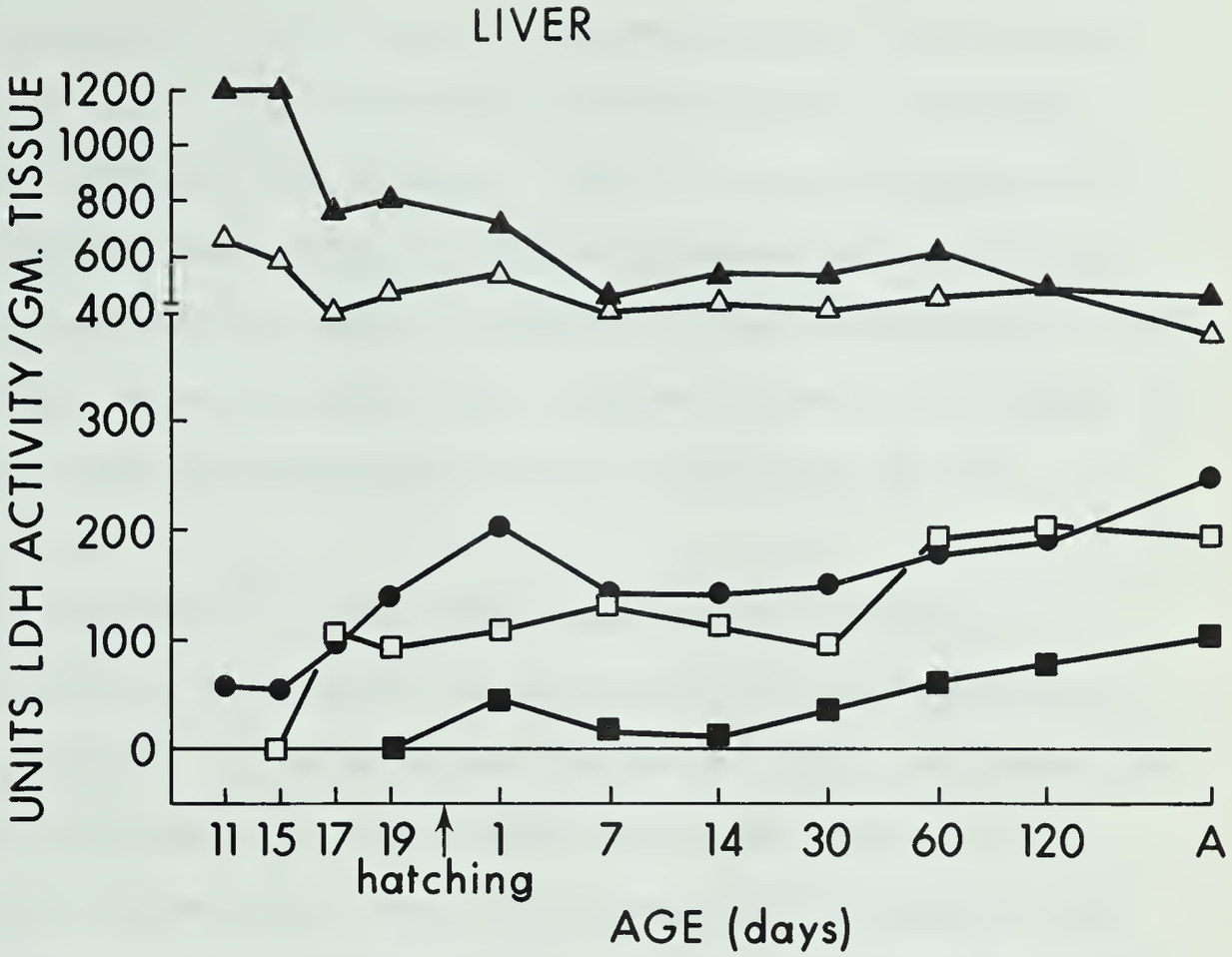
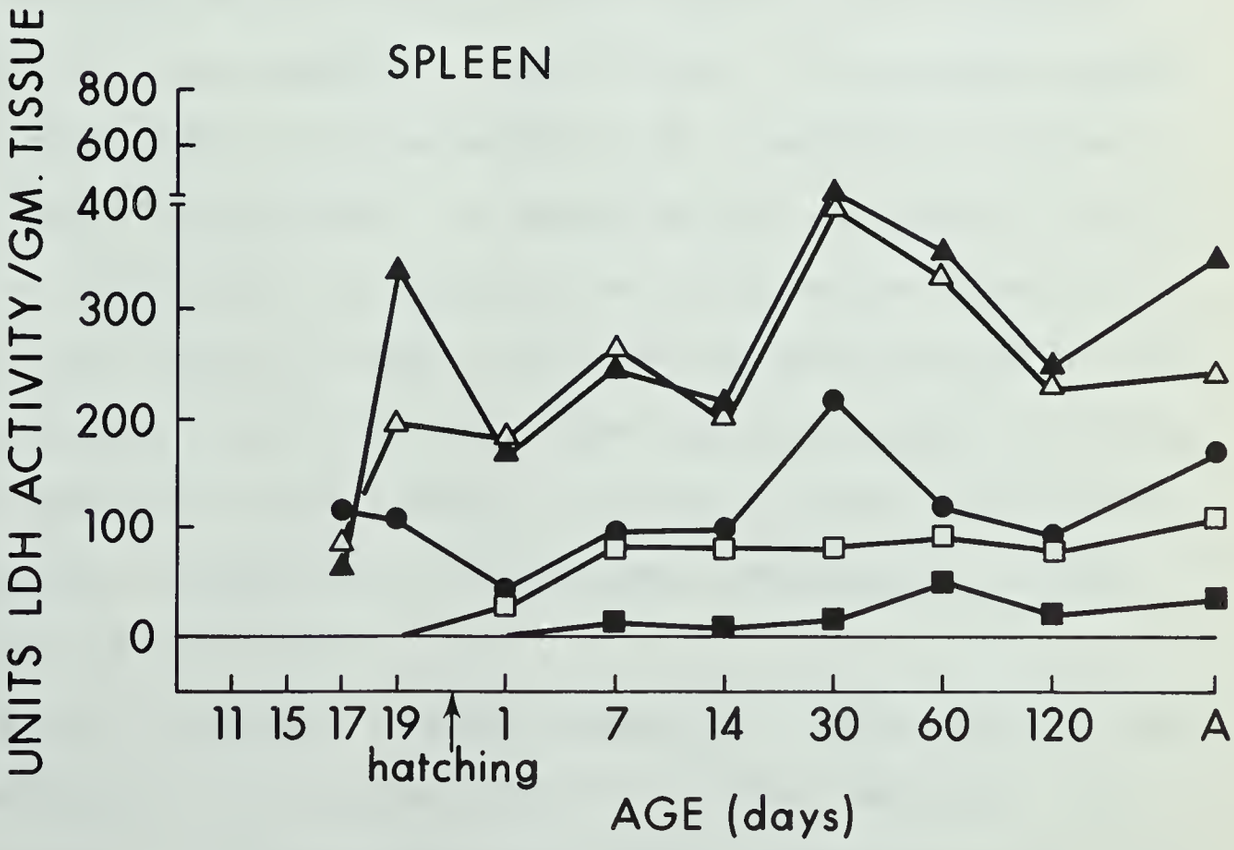

KIDNEY
UNITS LDH ACTIVITY/GM. TISSUE
1000
800
600
400
300
200
100
0
11 15 17 19 1 7 14 30 60 120 A
hatching
AGE (days)

Figure 16. The individual activities of the isoenzymes of liver and spleen during growth and development. The symbols are the same as given in Fig. 13 and the figure is directly comparable to Fig. 13.


LIVER
UNITS LDH ACTIVITY/GM. TISSUE
1200
1000
800
600
400
300
200
100
0
11 15 17 19 1 7 14 30 60 120 A
hatching
AGE (days)


SPLEEN
UNITS LDH ACTIVITY/GM. TISSUE
800
600
400
300
200
100
0
11 15 17 19 1 7 14 30 60 120 A
hatching
AGE (days)

than the separate activities of bursa LDH-1 and LDH-2 until 60 days of age. Since the total activities of the thymus and bursa LDH are very similar (Fig. 7), the difference is interpreted to mean that the thymus produces more of the B polypeptide (LDH-1 is BBBB, Markert). The rationale for the translation of activities into relative rates of synthesis of the consituent polypeptides of LDH is deferred to the 'Discussion'.

The decrease in bursa LDH after 60 days, evident in Figs. 7 and 13, parallels the involution of the bursa which begins at about 100 days in our chickens. This indicates that lymphoid involution is associated with a decrease of LDH activity. (As we shall see, it is possible to depress the LDH activity of the bursa without altering the weight of the organ. This suggests that the normal involution of the bursa may be preceded by a decrease of LDH activity, but we have not obtained data adequate to prove this.) The rather abrupt drop in bursa LDH is not accompanied by a decrease in thymus LDH, as seen for the adult (A) means of Figs. 7 and 13. The thymus of birds does not involute as fully as the bursa or as fully as the thymus of mammals so that the persistence of high thymus activity is no surprise. The slight increase in thymus activity after 120 days cannot, by itself, be taken as proof of a lymphoid hyperplasia of the thymus compensatory to the involution of the bursa.

Figure 14 compares two non-lymphoidal organs which reveal a preponderance of B type LDH (LDH-1 is BBBB, Markert). As seen in Figs. 7 and 14, brain LDH tends to decrease after 120

days in belated parallel to bursa LDH (Fig. 13). Conversely, heart LDH tends to increase after 120 days in parallel with thymus LDH (Fig. 13). However, the most obvious shift is seen in the embryo heart between the 11th and 19th days of incubation: LDH-4 appears and disappears and LDH-3 and LDH-2 decrease. The fact that LDH-1 increases rapidly at this time suggests that the transient increase of polypeptide A (LDH-4 is AAAB, Markert) at 15 days is accompanied by a great increase in polypeptide B (LDH-1 is BBBB). Since LDH-2 and LDH-3 decrease at the time LDH-4 disappears, we infer that the embryo heart undergoes a concomitant repression of A synthesis and a derepression of B synthesis.

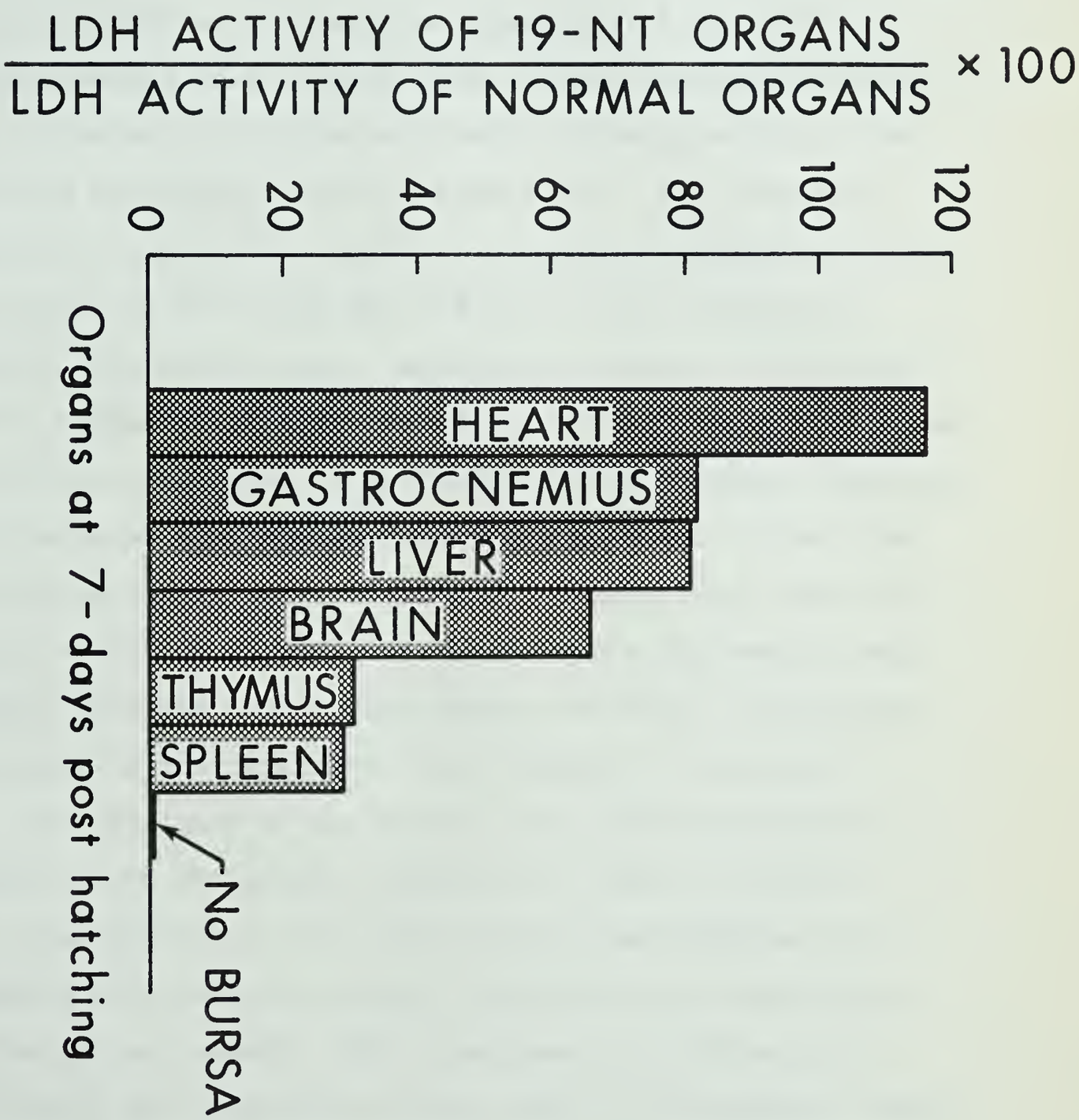
Figure 15 compares the individual activities of leg muscle/gastrocnemius with kidney. The gastrocnemius displays a unique elimination of LDH-1 which is complete at 120 days. This is accompanied by a marked increase of LDH-5, lesser increases of LDH-2 and LDH-4 and a slight decrease of LDH-3. When transformed to relative estimates of A and B polypeptides, these changes indicate that the decrease in B is approximately twice the increase in A, between 60 and 120 days. This suggests, more strongly than for heart, that a derepression of the synthesis of one of the polypeptides is accompanied by a repression of the other. The absence of LDH activity in adult gastrocnemius, excepting that due to LDH-5, reinforces this impression. Figure 8 indicates that the total activity of the gastrocnemius is rather constant throughout these changes, which further implies that the syntheses of A and B polypeptides are interlocked by an unknown reciprocal mechanism.

Figure 16 compares two organs which display all five isoenzymes. Both organs reveal a close parallel between LDH-1 and LDH-2 and a slight, progressive shift from B to A. These organs are distinctive for the variety of functions they perform: the liver, although largely composed of hepatic parenchyma, has multiple roles, and the spleen is a polygenetic mixture of erythropoietic, lymphopoietic, phagocytic, and other cells subserving diverse functions. It is no surprise that the two organs maintain a complex LDH pattern representing appreciable activities of all five isoenzymes.

IV. Effect of 19-nortestosterone Administration

In the first experiment, eggs were incubated five days and then injected with 0.63 mg of 19-nortestosterone in 0.1 ml of corn oil. Sham controls, which received corn oil only, and untreated controls were included. In all, 126 embryos and chicks were used. Organs were examined at 11, 15, 17, and 19 days of incubation and 1 and 7 days post-hatching. The percentile activities of the isoenzymes were not altered. That is, the isoenzymes are equally resistant to depression by 19-nortestosterone. The lymphoid tissues of the treated birds developed so slowly that it was not possible to do rate determinations of any of the lymphoid tissues before the seventh day post-hatching. The total activities at 7 days are given in Fig. 17 as percentiles of the controls. The sham and untreated controls do not differ and are grouped to obtain the '100%' control values. The 'treated' heart shows a slight increase in activity and the leg muscle and liver

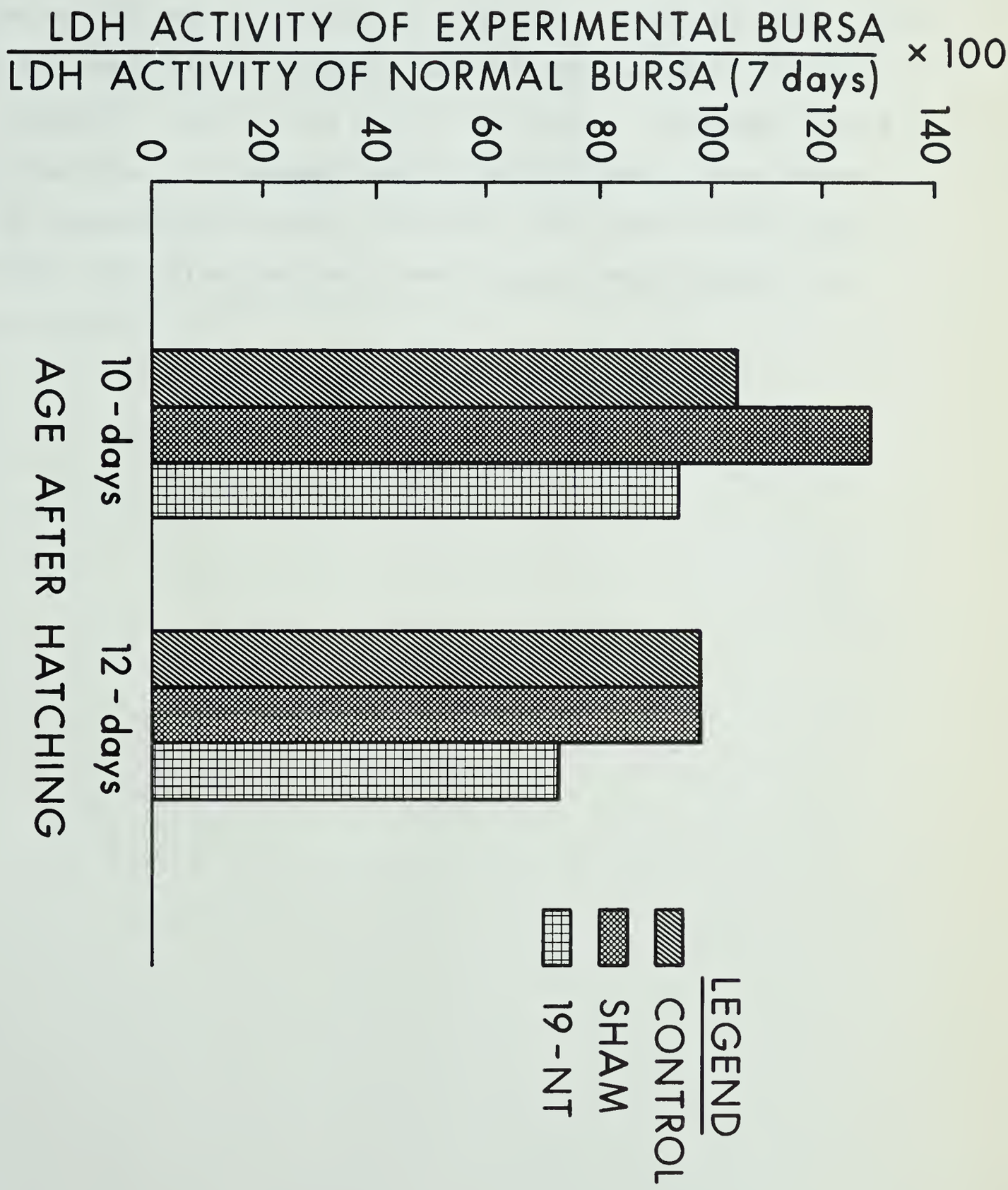
Figure 17. The effect of 19-nortestosterone on the LDH activity of seven organs of the chick, injected at five days of incubation. The dosage was 0.63 mg 19-nortestosterone in 0.1 ml of corn oil. All values presented are derived from activity measurements made on organs from 7-day chicks, and are expressed as the percentiles of the control values. The activities of the spleen and thymus are depressed as are their weights. The LDH activity of the brain is depressed, but not as much as the activities of the lymphoid tissues. 19-NT = 19-nortestosterone.


LDH ACTIVITY OF 19-NT ORGANS
LDH ACTIVITY OF NORMAL ORGANS
× 100
0
20
40
60
80
100
120
HEART
GASTROCNEMIUS
LIVER
BRAIN
THYMUS
SPLEEN
No BURSA
Organs at 7-days post hatching

show slight decreases. The brain shows a marked decrease and the spleen and thymus show profound decreases. The bursa of Fabricius did not develop and its activity could not be measured.

Since the early injection of 19-nortestosterone precludes the development of the bursa of Fabricius, 1 mg of 19-nortestosterone in 0.1 ml of corn oil was injected directly into the bursa epithelium and cavity of 7-day chicks. The injection was repeated on the eighth day. Six untreated controls, six corn oil controls, and six experimental chicks were killed on the tenth day and ten of each group were killed on the twelfth day. Neither the weight of the bursa nor the percentile activities of the isoenzymes were affected by the corn oil or the 19-nortestosterone. However, the corn oil stimulated the total activity on the tenth day and the 19-nortestosterone neutralized this stimulation. The corn oil had no effect on the total activity on the twelfth day at which time the 19-nortestosterone produced a significant depression of the total activity. Figure 18 gives the mean total activity of the control and treated bursas as percentiles of the normal activity at 7 days. The fact that a depression of total LDH activity was obtained with a lymphostatic hormone without a reduction in organ weight is presumptive evidence that a depression of LDH activity may precede the involution of the bursa. The moderate degree of hormonal depression of LDH three and five days after treatment, is not adequate to suggest that LDH depression is a primary step in the events leading to involution.

Figure 18. The effect of 19-nortestosterone on the LDH activity of the bursa of Fabricius of 10 and 12 day chicks, treated at 7 and 8 days. Chicks were injected with a dosage of 1 mg 19-nortestosterone per 0.1 ml of corn oil directed into the bursa epithelium. Values are expressed as percentiles of the LDH activity of a normal 7 day bursa. Activities were determined as outlined in 'Materials and Methods'. Involution of the bursa of Fabricius in response to 19-nortestosterone has not begun at 12 days, i.e. the weight of the bursa is unaffected.


LDH ACTIVITY OF EXPERIMENTAL BURSA
LDH ACTIVITY OF NORMAL BURSA (7 days)
× 100
0
20
40
60
80
100
120
140
10-days
12-days
AGE AFTER HATCHING
LEGEND
CONTROL
SHAM
19-NT

The transient stimulation of the LDH activity by corn oil should not be disregarded. An analagous stimulation of bursa weight has been reported (Aspinall, Meyer, and Rao, 1961) and our observation substantiates the importance of the corn oil control. Corn oil is one of the common 'innocuous' media for steroids. We suggest that it may include a trace amount of an unknown lymphogenetic stimulant, but this has not been tested. The effect may be a very general and indirect one, e.g. caloric.

DISCUSSION

> "The brightest flashes in the world of thought are incomplete until they have been proved to have their counterparts in the world of fact."
>
> -- John Tyndall

I. General

The increasing importance assigned to Immunology has prompted new studies of lymphoid tissues. This thesis was undertaken with the intent of establishing new directives and parameters for studies of the lymphoid activities basic to transplantation immunology. To this end we selected an enzyme activity essential to vertebrate life, accessible to limited means, instructive of techniques of broad application, and closely linked to the utilization of glucose; the metabolism of glucose is, in turn, closely linked to alterations of lymphoid tissue (Blecher, 1964). Ideally, and perhaps unavoidably, an approach of this sort must include nonlymphoid as well as lymphoid tissues so that enzyme characteristics peculiar to lymphoid tissue may be recognized. The enzyme activity selected, that of lactate dehydrogenase (LDH), serves the purpose: LDH is characteristic of viable vertebrate cells; its activity may be studied in simple extracts by fairly direct methods; it provides instruction in techniques applicable to many nicotinamide adenine dinucleotide (NAD) dehydrogenases; and it is fundamental to the interconversion of lactate and pyruvate, metabolites

which are central to the full utilization of glucose. Beyond these considerations, LDH occurs in several molecular forms, the synthesis of which may be influenced by steroid hormones (Kaplan, 1965), which alter the lymphoid tissues and affect the utilization of glucose. The inclusion of central nervous tissue in this study has led to the suggestion of a metabolic similarity between central nervous tissue and lymphoid tissue; a similarity by which they stand apart from some other tissues. But however interesting the results, the interpretation of them hinges on the validity of the methods used to obtain and collate them.

II. Validity of the Results

Two techniques were used to gather data on LDH. Both are based on the conversion of lactate to pyruvate in the presence of NAD, but the conditions of the reactions are quite different. The estimations of total LDH activity are derived from successive 15-second readings of the increased light absorption, due to the formation of $NADH_2$, which follows the addition of tissue extract to a solution of lactate and NAD: ambient temperature, 22°C $\pm$ 1°C; pH 9.0; and 0.02 M lactate. The reaction proceeds rapidly, passing through a maximal rate within two minutes and reaching a plateau within five minutes. The maximal rate is used to estimate the enzyme activity. The same reaction and conditions are used to estimate the LDH activity of all tissues. Since the tissue extracts contain unlike proportions of LDH isoenzymes, the estimations are summations of different

quantities of five kinds of enzymatically active molecules. What did we do to minimize distortion of the estimates due to disproportions of the five isoenzymes?

The activities of the isoenzymes are affected by the temperature, the pH, the concentration of substrate, and the quality of the coenzyme. The quantitative reaction is usually run by mixing pyruvate with $NADH_2$ and observing the decrease in optical density due to the enzymatic dehydrogenation of $NADH_2$; the reverse of our reaction. We did not estimate activity in this way because commercial $NADH_2$ is unreliable (Dolin, 1962), the reaction is very rapid and is best observed with automated equipment which we do not have, and the individual activities of the isoenzymes are differently and strongly affected by the concentration of pyruvate (Plagemann, Gregory, and Wroblewski, 1960b). This sets our work apart from most of the current quantitative work on the LDH isoenzymes. The use of lactate does not, however, void the question of substrate concentration. We selected a concentration which elicits maximal activity and we used a high pH which further ensures maximal activity, as reported for mammalian LDH (Bonavita, et al., 1964; Vesell, 1965a). The singular uncontrolled variable is temperature and this did not oscillate during the daily periods of the season when the estimations were made. Although the validity of our quantitative estimations is largely confined to comparisons within our own data and strict quantitation was never attempted, our estimates agree quite well with the literature.

The second method contrasts in many ways with the first.

A tissue extract is first submitted to electrophoresis on cellulose acetate. This separates the five LDH isoenzymes which are detected individually, not intermixed in solution as in the first method. Again, a concentration of lactate (0.019 M) is used which is near-optimal for all LDH isoenzymes. The pH is 7.4, which might tend to distort the estimates of individual isoenzyme activities were it not for the high temperature (37°C) of the reaction (Vesell, 1965a). Unlike the rate determination, this reaction proceeds for a fixed interval at the end of which the accumulated product is measured. The number of uncontrolled variables in this reaction is beyond our ability to estimate. Consequently, we devised a simple means of circumventing the uncertainty. We obtained a photometric scanner with a variable response control and found the setting which gave scans corresponding to activity estimates of the individual isoenzymes represented by the colored bands; the activity estimates were based on rate determinations of eluates.

The means of correcting for the unknown vagaries of the color reaction relies on a hidden assumption; that chicken and rabbit isoenzymes are congruous. Our data is collected from chicken tissues, but the standardization of the photometric scans was done with commercial rabbit muscle LDH. This has its reasons. Chicken LDH migrates very slowly and segregates sluggishly in comparison with mammalian LDH (Cahn, et al., 1962, Pesce, et al., 1964). After 1-1/2 hours migration (longer times are complicated by diffusion) chicken LDH-1 and LDH-5 may be less than 1.5 cm apart. It

is difficult to cut an unstained membrane so as to cleanly and reliably separate the chicken isoenzymes. It is much easier to do this with mammalian isoenzymes which may be dispersed over a distance several times greater. In fact, chicken LDH is the least suited to the technique.

Others have reported semi-quantitative scans of LDH, but the available reports deal with mammalian LDH (Regniers and Wieme, 1962; Barnett, 1964; Takamori, Hori, and Nishio, 1966). Barnett, who introduced the scanning of LDH on cellulose acetate, 'cleared' his strips with oil in order to make them semi-transparent. This is, we believe, a tricky procedure. The product of the reaction is soluble in many organic solvents, and is particularly soluble in those solvents most capable of clearing the membrane. We omitted clearing and scanned the dried membrane without further preparation. Such membranes keep their colors for many weeks. The use of dried membranes reduced the transmission of light which is not notably intense under any circumstance, with our apparatus. The low transmission of light prohibited the use of light filters and our scans would, presumably, be much better if we had been able to use a more intense light source and narrow-band interference filters.

Neither Barnett, nor any of the other authors who have reported scans of LDH have, to our knowledge, confirmed the validity of the scans by separate activity estimations of eluates (Barnett, 1964; Takamori, Hori, and Nishio, 1966). This applies to Wieme's scans of $NADH_2$ fluorescence in agar gels as well (Regniers and Wieme, 1962). In fact, our selection

of cellulose acetate membranes as the electrophoretic medium was based on the expectation of easy elution; an expectation confirmed by the recovery of 85 to 90% of rabbit muscle LDH activity. For comparison, Plagemann, Gregory, and Wroblewski (1960b) recovered 55% of the total activity from starch gel. A recovery of 55% would not, in our opinion, be a reliable basis for the survey reported here.

There are several other ways of estimating the activities of individual isoenzymes. All are founded on one assumption which sets them apart from the scanning methods. The scanning methods depend on the electrophoretic separation of the isoenzymes which are estimated separately in situ. These other methods depend on the inhibition of either the A or the B polypeptide's contribution to the total activity. The inhibition of each isoenzyme depends on its composition and is proportional to the number of affected polypeptides it contains (Plagemann, Gregory, and Wroblewski, 1960b). The total inhibition is an estimate of the amount of the affected polypeptide in the tissue extract and represents the sum of the affected polypeptides of all the isoenzymes. The underlying assumption is this: the proportion of the total activity due to each isoenzyme may be predicted from the estimate (by inhibition) of the total A and B polypeptides present, through expansion of the binomial to the fourth power. This has been done for chicken LDH by Lindsay (1963). There is a remarkable lack of formal proof for this assumption when applied to tissue extracts. The fact is that tissue extracts not infrequently contain more than five isoenzymes,

some of which contain polypeptides other than A and B. The presence of additional isoenzymes need not confuse the scanning method provided the additional isoenzymes have unique electrophoretic migrations. Admittedly, inhibition and scanning are complementary methods and the present work would be more satisfying if time permitted the systematic testing of one or more inhibitors.

The scans obtained from independent samples of identical age are quite similar. The same cannot be said for the rate determinations which are the source of troublesome variation. Inspection of the graphs suggests that the serrate character of the lines is partly artifactual and that more data would rearray the means in linear and curvilinear modes. We have not found better representations in the LDH literature which includes many smooth graphs, the bases for which are not entirely clear.

The principal isoenzymes in the rabbit LDH used for standardization are LDH-4 and LDH-5. A 100-fold range of dilution indicates that scans of 50 to 500 units/ml of any one isoenzyme are reliable, but scans of lower activities are suspect. Wilkinson (p. 38, 1965) indicates that scans of weak bands may be exaggerated. Nevertheless, these weak bands can be useful. Figure 10 shows that the young embryo heart possesses appreciable amounts of polypeptide A, as indicated by the presence of LDH-4. The transitory presence of LDH-4 in the chicken heart is not described in the literature. This small discovery is the more interesting

because the embryo pig heart also contains A-type LDH which disappears with further development. In short, with the present technique one of the troubling points of difference between the mammal and the bird is lessened. In this regard, it may actually help if the scans exaggerate weak bands which otherwise might go unnoticed.

III. Enzymes and Development

Many studies have been made on the localization of enzymes and their changes in activity during development. Steinbach and Moog (1945) found that adenyltriphosphatase activity increased in the large granules of embryonic chick liver. Glutamic dehydrogenase, lactic dehydrogenase, and malic dehydrogenase activities have been shown by Solomon (1957, 1958) to increase in liver homogenates of developing chick embryos at about fifteen days incubation. A summary by Phillip and Vesell (1962) further reports that the early chick embryo contains low phosphatase activity, relatively high peptidase activity, and very high concentrations of cytochrome oxidase and succinoxidase. We have also found characteristic changes in the activity of lactate dehydrogenase in the chicken. Solomon (1959) reports that the LDH activity of embryonic chick liver reaches a maximum at about 15 days incubation and then drops to more than one half the level by the time of hatching. Strittmatter (1965) reports the same effect. We observed a drop, but it was not so abrupt. We also observed an initial increase in heart LDH at about 15 days incubation while the brain, thymus, and bursa of

Fabricius levels do not change much through development and growth. Kidney shows a marked increase immediately after hatching and then returns to normal levels by the first week of age. Leg muscle/gastrocnemius and spleen show slight increases in LDH activity throughout the developmental stages we have studied. It is difficult to attribute these fluctuations of enzymatic activity to any changes concerned with the differentiating organ or tissue, but these changes in enzymatic activity must reflect metabolic changes.

What seems to be more important, however, is the accompanying changes in isoenzyme patterns. The patterns of distribution of LDH isoenzymes from embryonic tissues are not always identical with their adult counterparts. The first observation of such differences was that of Markert and Møller in 1959 who detected a larger number of LDH components in embryonic pig heart than in adult heart. They reject the suggestion that the differences between the adult and embryonic patterns may be due to changes in cell population since the observed population changes during development do not appear to be sufficiently great to account for such a possibility. They, thus, prefer to consider the isoenzyme pattern of a tissue as a parameter of the state of differentiation of its cells. Markert and Ursprung (1962) further view ontogenetic changes in LDH isoenzyme patterns in the mouse. While most of the embryonic tissues initially contain principally LDH-5, as development proceeds LDH activity is gradually shifted toward the more anodal LDH-1 end of the spectrum. In the

skeletal muscle of this species, LDH-5 predominates in the embryonic forms, but measurable amounts of other isoenzymes occur. In the adult form, LDH-1, LDH-2, and LDH-3 are usually not detectable. The rates of change of these LDH isoenzyme patterns vary from organ to organ.

In the rat heart, changes during development resemble those occurring in the mouse (Kaplan and Ciotti, 1961). LDH-5 appears first but soon after birth, LDH-1 replaces LDH-5 as the most abundant form. Similar changes have been observed by Bonavita, et al. (1964) in the developing rat brain.

The difference in the degree of inhibition of LDH isoenzymes by excess pyruvate appears to be of considerable metabolic significance. Since the reduction of pyruvate to lactate by LDH-1 is strongly inhibited by quite low concentrations of pyruvate, it has been suggested that the rapid accumulation of lactate could not occur in a tissue rich in this isoenzyme, and complete oxidation of glucose via the citric acid cycle is therefore to be expected. LDH-5 on the other hand, functions more efficiently when exposed to concentrations of pyruvate inhibitory to LDH-1 and is inhibited only by much higher concentrations. It appears that tissues rich in LDH-5 then, would allow for the rapid conversion of pyruvate into lactate and hence, the establishment of an oxygen debt under anaerobic conditions (Cahn, et al. 1962). Thus, in the rat and mouse, anaerobic glycolysis leading to the accumulation of lactate appears

to be the principal metabolic pathway *in utero* and it has been suggested that excess lactate may be removed via the placenta. However, Vesell (1965a) has recently shown that purified LDH-1 and LDH-5 are very similar in susceptibility to inhibition by substrate when tested at physiological temperature (37°C). These results are incompatible with the theory that differences in degree of isoenzyme inhibition by substrate have resulted in the predominance of LDH-5 in anaerobic tissues and the predominance of LDH-1 in aerobic tissues.

Furthermore, not all mammals have LDH-5 as the dominant isoenzyme in the embryo. Pfleiderer and Wachsmuth (1961) show that the human embryo contains all five lactate dehydrogenase isoenzymes, LDH-3 being the most abundant. During development the proportions of LDH-1 and LDH-5 increase so that normal tissue patterns appear soon after birth. The relative proportions of the various isoenzymes in embryonic skeletal muscle and heart muscle are close to the ideal binomial distribution expected as the result of A and B monomers. Hinks and Masters (1964) also demonstrate that the 'B' polypeptide is the predominant subunit in bovine and ovine embryos.

The chicken embryo also has LDH-1 as its dominant isoenzymic form. With the aid of a quantitative complement-fixation technique, Cahn, *et al*. (1962) observed that the lactate dehydrogenase of breast muscle from a 6 day chicken embryo was identical with that of an adult chicken heart,

whereas that of an 8 day chick migrates like normal adult skeletal muscle. We did not find as rapid a shift to the LDH-5 adult form in another skeletal muscle, the gastrocnemius, as did these workers for breast muscle. Phillip and Vesell (1962) compared the isoenzyme patterns obtained by starch-gel electrophoresis of chick embryo liver, muscle, and heart at various stages of development. They found that at 8 days after fertilization, LDH-1 was the most abundant isoenzyme of all three organs but that traces of LDH-2 and LDH-3 were also present in smaller amounts. Liver LDH-1 is accompanied by two faster migrating bands of low activity. During development, they report a gradual shift of activity from the anodal to the cathodal isoenzymes, and appreciable amounts of LDH-4 and LDH-5 are detectable in adult liver and muscle. On the other hand, they observed little change in the isoenzyme pattern of the developing heart. Our results agree with these findings excepting the shift to LDH-1 which we observe in the heart, but they do not report. Spleen and kidney behave like liver in the gradual shift of activity toward the cathodal isoenzymes but the rates differ slightly. Phillip and Vesell (1962) do not define which muscle they worked on, but in gastrocnemius the pattern shifts completely to almost pure LDH-5 in the adult chicken.

We did not find the two bands anodal to LDH-1 reported by Phillip and Vesell (1962) and also by Croisille (1964) in our strains of chickens but we have seen them in kidney,

liver, and spleen of commercial chickens. Croisille (1964) also reports two even faster 'E' bands in embryonic liver and kidney of embryos of 10 and 15 days of incubation which, however, disappeared before hatching. We have never observed these bands in any of the chicken embryos we have studied. These 'E' bands do not appear to be the 'nothing dehydrogenase' reported by Shaw and Koen (1965). Rather, these extra bands may account for the very high levels of LDH activity in embryonic liver reported by Solomon (1959) and Strittmatter (1965). The fact that brain, thymus, and bursa of Fabricius isoenzyme patterns change little during development is of considerable interest and shall be discussed later.

Further information on LDH's in chicken tissues is provided by Maisel, Kerrigan, and Syner (1965) on a study of the ontogeny of LDH isoenzymes in the lens of the white leghorn chick with starch-gel electrophoresis. LDH-1 appears to be the major embryonic form and the more cathodal forms appear progressively through development. They also show four more bands anodal to LDH-1 in the adult lens. In all, they show a total of nine different isoenzymes, none of which are equivalent to the occasional occurrence of an LDH-6 in the liver and spleen of adult chickens. However, our LDH-6 is equivalent to Nebel and Conklin's (1964) '5b' isoenzyme in chicken liver. A more recent report by Maisel and Alcorn (1966) shows that LDH-1, LDH-2, and LDH-3 were the most predominant isoenzymes in the chick cornea

between 7 and 12 days of embryonic development. From day 14 to hatching there is an increase in activity of LDH-4 and LDH-5 so that in the newborn chick, LDH-1 to LDH-5 show equal intensity of staining. With increasing age after hatching, LDH-4 and LDH-5 exceed the activity found in LDH-1-3.

In summary, an ontogenetic study of this form requires a comprehensive range of tissue specimens to accurately establish the direction of isoenzyme redistribution. Some slight individual differences do occur from time to time and differences between strains of the same species do exist.

The study of the redistribution of LDH isoenzyme activity is particularly interesting. In 1964 Dawson, Goodfriend, and Kaplan showed the changes in activity of gastrocnemius of the rabbit to be due almost entirely to an increased synthesis of the M-type (A, Markert) subunit. Similarly in heart, they showed it to be due almost entirely to the synthesis of the H-type (B, Markert) subunit. That is, the genes for the two types of subunits can be regulated independently. The view that genes controlling the synthesis of LDH isoenzymes can be regulated is not without support. Goodwin and Sizer (1965) recently did a study which showed that histone, when added at low concentrations to cultures of embryonic chicken brain tissue, causes an inductive response in lactic dehydrogenase activity, whereas at higher concentrations of histone, the response is repressive. The control is shown to operate by altering protein synthesis. These authors suggest that the observed inductive response of

LDH to low concentrations of histone may be explained as a secondary result due to the repression of another genetic locus which directs the synthesis of either an aporepressor or a corepressor of the LDH gene or genes (since isoenzymes of this enzyme occur in chick brain). This requires that the histones have a higher affinity for this locus than for either or both of the LDH genes so that it is most affected when there is little histone present. As the histone concentration is increased, repression will be increased to include more loci, with the result that at some concentration, the LDH genes will themselves be repressed. At low concentrations, the histones first repress some key loci whose activity tends to control LDH syntheses, thereby releasing the LDH loci so that the amount of protein synthesis is increased, i.e. the LDH genes may be derepressed.

We do not dispute the fact that the genes for the synthesis of lactate dehydrogenases can be regulated. However, the changes in A and B seem to be reciprocal, not independent. In the chicken gastrocnemius it appears that LDH-5 synthesis is associated with a suppression of LDH-1 while in the heart, the increased percentage of LDH-1 is associated with a suppression of LDH-4. It is worth noting here that we determine individual isoenzymes and Kaplan and his colleagues determine the activity of A and B polypeptides. The underlying assumptions for these two approaches are quite different.

It seems that from the rest of the data, the general interpretation that the isoenzymes represent random aggregation

of subunits and fit a unimodal binomial distribution is correct. Throughout all the organs except gastrocnemius, the B-type subunit seems to be in greatest quantity and with the exception of heart, there appears to be a general parallelism between LDH-1 and LDH-2. The initial inversion of LDH-1 and LDH-2 in bursa, thymus, and spleen cannot be explained at this time. However, in the spleen, the initial drop of LDH-3 occurs at about the time of the shift of this organ from erythropoiesis to lymphopoiesis.

Since heart seems to be an exception to the general concept of a binomial distribution of isoenzymes, it may be that the genetic mechanism for B-type LDH may be determined by mitochondrial DNA. On the other hand, most of the A-type and a bit more of the B-type may be determined by chromosomal DNA. This may appear in the extra-mitochondrial cytoplasm and will be synthesized entirely separately from LDH in the mitochondrion. The fact that there is LDH intramitochondrially is not without support. Walker and Seligman (1963) used formalin fixation of sections to provide unequivocal evidence for intramitochondrial staining for LDH in rat hepatic and acinar cells. Earlier, Novikoff and Masek (1958) found the same thing when using formal-calcium fixed sections of rat heart muscle. The technique in these two studies depends on conditions favoring mitochondrial permeability to permit penetration by substrate, coenzyme, and ditetrazolium salt without, at the same time, permitting leakage of the dehydrogenases. Vesell (1965b) has also made the suggestion

that since human platelets, lens fibres, and mature erythrocytes lack a nucleus and have only faint traces of LDH-5, there may be an association between LDH-5 and the cell nucleus. Thus, much or all of the LDH in these cells may be of mitochondrial origin. Conversely, some authors report that all LDH is extra-mitochondrial (Solomon, 1959).

This brings us to another aspect concerned with the brain, and the two lymphoid organs, the thymus and the bursa of Fabricius. They are all similar in that they lack LDH-4 and LDH-5 (or have it in much smaller quantities than most tissues) and do not change much during development. Further, they have relatively low levels of activity. A comparison of these facts suggests that the lower activities of the lymphoid tissues are due, at least in part, to very low levels or absence of LDH-4 and LDH-5. The absence or near absence of these forms of LDH is presumably due to non-synthesis in the lymphoid tissues. The non-detection of LDH-4 and LDH-5 is probably not due to selective and excessive destruction, since homogenates of lymphoid tissues do not affect the activities of LDH-4 and LDH-5 from other tissues. The non-detection of LDH-4 and LDH-5 is probably not due to selective and rapid leakage of LDH-4 and LDH-5 from lymphoid tissues. The possibility of selective leakage, like the possibility of selective destruction is difficult to exclude, but the historically important and best known instances of leakage or secretion have been traced to cells rich, not poor, in the relevant forms of LDH. We know of no general

evidence of rapid leakage from cells poor in these forms of LDH. We infer that the effective absence of LDH-4 and LDH-5 from the primary lymphoid organs is due to non-synthesis or trivial synthesis.

There are two foreseen implications of this kind of information. One, it provides a basis of comparison of lymphoid tissues with aerobically glycolytic tissues such as brain. The LDH pattern of the chicken brain is very similar to that of the primary lymphoid tissues. It is interesting to note, further, that the profound effects of 19-nortestosterone on the lymphoid tissues are accompanied by a quantitative suppression of the LDH activity of the brain (Fig. 17). Meyer, Rao, and Aspinall (1959) indicate that it prevents the bursa of Fabricius from developing at all which is true in our experiment as well. However, in our second experiment, we have shown that this steroid also suppresses the total LDH activity of bursa of Fabricius. It has no effect on bursa size. Figure 18, however, shows a stimulatory effect of the corn oil at 10 days which then drops off by 12 days of age. Aspinall, Meyer, and Rao (1961) found that the corn oil stimulates the weight of the bursa of Fabricius and thymus relative to untreated controls when eggs were injected at 5-days incubation. The gross sequential aspects of these effects are clear enough, but the implications demand a better assignment of the effect to cell type than we are able to provide at this time. For the present, we note that the androgen does not alter the LDH pattern, but

it does suppress the LDH activity of some tissues poor in LDH-4 and LDH-5.

The second implication of the simple embryonic LDH pattern of the primary lymphoid tissues is more important. If lymphoidal cells do synthesize relatively small amounts of LDH-4 and LDH-5, this must mean that the genetic message for this synthesis is not activated and remains suppressed. That is to say, the acquisition of lymphoid potencies is associated with the hardening of a repression which might otherwise be overcome. The converse, that lymphopoiesis involves the reacquisition of a repression previously lost, cannot be tested in the chicken embryo by studies of LDH. Useful pursuit of both implications seem to require techniques which delineate the LDH patterns of individual cells so that alterations associated with specific differentiations may be identified. It is interesting to note that Markert and Faulhaber (1965) have openly questioned the physiological significance of LDH isoenzyme patterns and have implied that they may possess no physiological significance. Relative to the intent of this thesis, it is appropriate to put the question differently: Do the different LDH isoenzymes play a causal role in differentiation or are they, at best, only consequences of differentiation? We suggest that the latter assessment is closer to the mark. The LDH pattern may be a useful reference, but it is likely that a more useful insight into the differentiation of the lymphoid tissue, and its modification by hormones, will be found in the lysosomal

enzymes, proteases, and nucleases (Maor and Alexander, 1965), of lymphoid and other cells.

IV. Hormones and LDH Isoenzymes

Kaplan (1965) has described a stimulus of M (A) polypeptide synthesis by androgen in seminal vesicles. In contrast, we report a depression of H (B) and M (A) by androgen in brain and lymphoid tissue. Thus, it would seem that LDH polypeptides bring us no closer to an understanding of the initial action of sex hormones than do the more classical observations of organ changes. The changes in LDH seem to be just one more expression of the differentiated cell's selective response to the hormone. The basic enigma of why a hormone affects only some cells remains undented. In any case, specific responses to sex hormone do not seem to be mediated by specific kinds of LDH and LDH isoenzymes cannot be advanced as the sites of action of the androgens which suppress lymphoid and immune activities, although they are sensitive indicators of the suppression; much more rapid and sensitive indicators than a decrease in lymphoid weight or a weakening of an immune response.

SUMMARY

1. Using the techniques of electrophoresis on cellulose acetate and dehydrogenase cytochemistry coupled with densitometric scans correlated with elution experiments, the lactate dehydrogenase (LDH) isoenzyme patterns of several organs were studied during the growth and development of the chicken. When the percentile values from densitometry were converted to unit measurements based on activity estimates from spectrophotometry (using optimal concentrations of lactate), data on quantitative changes in LDH isoenzymes during the growth and development of the chicken were also obtained.
2. LDH has been shown to exist in six electrophoretically distinct varieties or isoenzymes in the chicken.
3. The pattern of isoenzyme distribution in embryonic tissues of the chicken differs from that of the adult. LDH-1 is the major form in embryonic tissues but the pattern shifts to LDH-5 in the gastrocnemius of the adult chicken. Similarly, liver, spleen, and kidney gradually acquire the cathodal LDH's, LDH-4 and LDH-5, but without the loss of the anodal ones. Brain, thymus, and bursa of Fabricius differ in that they are characterized by only the anodal LDH's, LDH-1-3. They do not change much during development. Heart shows an increasing predominance of LDH-1 throughout development.

4. The suggestion that LDH-5 synthesis in the gastrocnemius is associated with a suppression of LDH-1 and that the LDH-1 synthesis in heart is associated with a suppression of LDH-4 is discussed.
5. The brain, and the two lymphoid organs, the thymus and the bursa of Fabricius are all similar in that they lack LDH-4 and LDH-5 (or have it in very low quantities) and the LDH patterns of these organs do not change much during development. Further, the profound effects of 19-nortestosterone on the lymphoid tissue are accompanied by a quantitative suppression of the LDH activity of the brain. The implications of this information are discussed.

LITERATURE CITED

Appella, E., and Markert, C. L. 1961. Dissociation of lactate dehydrogenase into subunits with guanidine hydrochloride. Biochem. Biophys. Res. Commun. 6: 171-176.

Aspinall, R. L., Meyer, R. K., and Rao, M. A. 1961. Effect of various steroids on the development of the bursa Fabricii in chick embryos. Endocrinology. 68:944-949.

Barnett, H. 1964. The staining of lactic dehydrogenase isoenzymes after electrophoretic separation on cellulose acetate. J. Clin. Path. 17:567-570.

Blecher, M. 1964. Effects of cortisol on the metabolism of glucose by lymphoid tissue. J. Biol. Chem. 239:1299-1300.

Bonavita, V., Ponte, F., and Amore, G. 1964. Lactate dehydrogenase isoenzymes in the nervous tissue IV: An ontogenetic study of the rat brain. J. Neurochem. 11:39-47.

Cahn, R. D., Kaplan, N. O., Levine, L., and Zwilling, E. 1962. Nature and development of lactic dehydrogenases. Science. 136:962-969.

Croisille, Y. 1964. Formes multimoléculaires de la déshydrogénase lactique chez le poulet: demonstration de l'existance de formes transitories chez l'embryon. Compt. Rend. Acad. Sci. 258:2214-2217.

Dawson, D. M., Goodfriend, T. L. and Kaplan, N. O. 1964. Lactic dehydrogenases: Functions of the two types. Science. 143:929-933.

Dolin, M. I. 1962. Effect of near-ultraviolet irradiation on the peroxide content of solutions of oxidized or reduced diphosphopyridine nucleotide. Biochem. Biophys. Acta. 63:219-221.

Elvehjem, C. A. 1949. p. 1-6. In H. A. Lardy, ed., Respiratory enzymes. Burgess Publishing Company, Minneapolis.

Farber, E., Sternberg, W. H., and Dunlop, C. E. 1956a. Histochemical localization of specific oxidative enzymes. I. Tetrazolium stains for diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase. J. Histochem. Cytochem. 4:254-265.

Farber, E., Sternberg, W. H., and Dunlop, C. E. 1956b. Histochemical localization of specific oxidative enzymes. III. Evaluation studies of tetrazolium staining methods for diphosphopyridine nucleotide diaphorase, triphosphopyridine nucleotide diaphorase, and the succinic dehydrogenase system. J. Histochem. Cytochem. 4:284-294.

_____ 1956c. Histochemical localization of specific oxidative enzymes. V. The dissociation of succinic dehydrogenase from carriers by lipase and the specific histochemical localization of the dehydrogenase with phenazine methosulfate and tetrazolium salts. J. Histochem. Cytochem. 4:357-362.

Goodwin, B. C. and Sizer, I. W. 1965. Histone regulation of lactic dehydrogenase in embryonic chick brain tissue. Science. 148:242-244.

Hinks, M. and Masters, C. J. 1964. Developmental changes in ruminant lactic dehydrogenase. Biochemistry. 3:1789-1791.

Horecker, B. L. and Kornberg, A. 1948. The extinction coefficients of the reduced band of pyridine nucleotides. J. Biol. Chem. 175:385-390.

Jerchel, D. and Möhle, W. 1944. Die Bestimmung des Reduktionspotentials von Tetraloziumverbindungen. Chem. Ber. 77:591-601.

Kaplan, N. O. 1965. Effect of hormones and environmental factors on lactic dehydrogenases. J. Cellular Comp. Physiol. 66:1-10.

Kaplan, N. O. and Ciotti, M. M. 1961. Evolution and differentiation of dehydrogenases. Ann. N. Y. Acad. Sci. 94:701-722.

Krebs, E. G. 1953. Yeast glyceraldehyde-3-phosphate dehydrogenase I. Electrophoresis of fractions precipitated by nucleic acid. J. Biol. Chem. 200:471-478.

Lakon, G. 1939. Das Schwinden der Keimfähigkeit der Samen, insbesondere der Getreidefrüchte. Ber. dtsch. bot. Ges. 57:191-203.

Lindsay, D. T. 1963. Isozymic patterns and properties of lactate dehydrogenase from developing tissues of the chicken. J. Exp. Zool. 152:75-89.

Maisel, H., Kerrigan, M. and Syner, F. 1965. The ontogeny of lactate dehydrogenase in the chick lens. Invest. Ophth. 4:262-271.

Maisel, H. and Alcorn, S. 1966. The ontogeny of lactate dehydrogenase (LDH) in the chick cornea. Anat. Rev. 154:382.

Maor, D. and Alexander, P. 1965. Abscopal stimulation of the thymus of rats by exposure of the head to X-rays. Nature. 205:40-42.

Markert, C. L. 1962. Isozymes in kidney development, p. 54-63. In J. Metcoff, ed., Hereditary, developmental, and immunological aspects of kidney disease. Northwestern University Press, Evanston, Ill.

_____ 1963. Lactate dehydrogenase isozymes: Dissociation and recombination of subunits. Science. 140:1329-1330.

Markert, C. L. and Møller, F. 1959. Multiple forms of enzymes: Tissue, ontogenetic, and species specific patterns. Proc. Nat. Acad. Sci. U. S. 45:753-763.

Markert, C. L. and Ursprung, H. 1962. The ontogeny of isozyme patterns of the mouse. Develop. Biol. 5:363-381.

Markert, C. L. and Faulhaber, I. 1965. Lactate dehydrogenase isozyme patterns of fish. J. Exp. Zool. 159:319-322.

Mattson, A. M., Jensen, C. V. and Dutcher, R. A. 1947. Triphenyltetrazolium chloride as a dye for vital tissues. Science. 106:294-295.

Meyer, R. K., Rao, M. A. and Aspinall, R. L. 1959. Inhibition of the development of the bursa of Fabricius in the embryos of the common fowl by 19-nortestosterone. Endocrinology. 64:890-897.

Nebel, E. J. and Conklin, J. L. 1964. Development of lactic dehydrogenase isozymes in the chick embryo. Proc. Soc. Exp. Biol. Med. 115:532-536.

Neilands, J. B. 1952. Lactic dehydrogenase of heart. I. Purity, kinetics,and equilibria. J. Biol. Chem. 199:373-381.

Nisselbaum, J. S. and Bodansky, O. 1959. Reactions of lactic dehydrogenase from various rabbit organs with antirabbit muscle lactic dehydrogenase. J. Biol. Chem. 234:3276-3280.

Nisselbaum, J. S. and Bodansky, O. 1961. Immunological studies of functionally similar enzymes. Ann. N. Y. Acad. Sci. 94:970-987.

Novikoff, A. B. and Masek, B. 1958. Survival of lactic dehydrogenase and DPNH-diaphorase activities after formol-calcium fixation. J. Histochem. Cytochem. 6:217.

Pesce, A., McKay, R. H., Stolzenbach, F., Cahn, R. D. and Kaplan, N. O. 1964. The comparative enzymology of lactic dehydrogenases. I. Properties of the crystalline beef and chicken enzymes. J. Biol. Chem. 239:1753-1761.

Pfleiderer, G. and Wachsmuth, E. D. 1961. Alters-und funktionsabhängige Differenzierung der Lactatdehydrogenase menschlicher Organe. Biochem. Z. 334:185-198.

Phillip, J. and Vesell, E. S. 1962. Sequential alterations of lactic dehydrogenase isozymes during embryonic development and in tissue culture. Proc. Soc. Exp. Biol. Med. 110:582-585.

Plagemann, P. G. W., Gregory, K. F. and Wroblewski, F. 1960a. The electrophoretically distinct forms of mammalian lactic dehydrogenase. I. Distribution of lactic dehydrogenases in rabbit and human tissues. J. Biol. Chem. 235:2282-2287.

_____ 1960b. The electrophoretically distinct forms of mammalian lactic dehydrogenase. II. Properties and inter-relationships of rabbit and human lactic dehydrogenase isozymes. J. Biol. Chem. 235:2288-2293.

Regniers, P. and Wieme, R. J. 1962. The lactate dehydrogenase isoenzyme pattern of human cells maintained in long-term culture. Arch. int. Pharmacodyn. CXL: 409-415.

Shaw, C. R. and Koen, A. L. 1965. On the identity of "nothing dehydrogenase". J. Histochem. Cytochem. 13:431-433.

Shelton, E. and Schneider, W. C. 1952. On the usefulness of tetrazolium salts as histochemical indicators of dehydrogenase activity. Anat. Rec. 112:61-81.

Smithies, O. 1955. Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. Biochem. J. 61:629-641.

Solomon, J. B. 1957. Glutamic dehydrogenase in the developing chick embryo. Biochem. J. 66:264-270.

_____ 1958. Lactic and malic dehydrogenases in the developing chick embryo. Biochem. J. 70:529-535.

_____ 1959. Changes in the distribution of glutamic, lactic, and malic dehydrogenases in liver cell fractions during development of the chick embryo. Develop. Biol. 1:182-198.

Steinbach, H. B. and Moog, F. 1945. Localization of adenyltriphosphatase in cytoplasmic granules. J. Cellular Comp. Physiol. 26:175-183.

Strittmatter, C. F. 1965. Studies on avian xanthine dehydrogenases: Properties and patterns of appearance during development. J. Biol. Chem. 240(6):2557-2564.

Takamori, Y., Nori, Y. and Nishio, K. 1966. Lactic dehydrogenase and radiation-induced lymphoid leukaemia in mice. Z. Naturforschg. 21b:352-357.

Tsou, K. C., Cheng, C. S., Nachlas, M. M. and Seligman, A. M. 1956. Syntheses of some p-Nitrophenyl substituted tetrazolium salts as electron acceptors for the demonstration of dehydrogenases. J. Am. Chem. Soc. 78:6139-6144.

Vesell, E. S. 1965a. Lactate dehydrogenase isozymes: Substrate inhibition in various human tissues. Science. 150:1590-1593.

_____ 1965b. Lactate dehydrogenase isozyme patterns of human platlets and bovine lens fibres. Science. 150:1735-1737.

Walker, D. G. and Seligman, A. M. 1963. The use of formalin fixation in the cytochemical demonstration of succinic and DPN- and TPN- dependent dehydrogenases in mitochondria. J. Cell. Biol. 16:455-469.

Webb, E. C. 1964. Nomenclature of multiple enzyme forms. Nature. 203:821.

Wieland, T. and Pfleiderer, G. 1957. Nachweis der Heterogenitat von Milchsaure-dehydrogenases verscheidenen Ursprungs durch tragerelecktrophorese. Biochem. Z. 329:112-116.

Wilkinson, J. H. 1965. Isoenzymes. E. & F. N. Spon Ltd., London. 158 p.

APPENDIX I

LDH ACTIVITY ASSAYS AND ISOENZYME ANALYSES OF ORGANS OF B2B14 CHICKENS

CODE

COL 2-4 AGE
COL 6 SEX CODE 1=MALE, CODE 2=FEMALE
COL 8 ORGAN CODE 1=BRAIN, 2=BURSA, 3=HEART, 4=KIDNEY, 5=LIVER
6=SPLEEN, 7=LEG MUSCLE/GASTROCNEMIUS, 8=THYMUS
COL 11-14 TOTAL ACTIVITY
COL 17-20 LDH 1
COL 23-26 LDH 2
COL 29-32 LDH 3
COL 35-38 LDH 4
COL 41-44 LDH 5

TOTAL ACTIVITY=LDH UNITS/GM TISSUE
ISOENZYME INDIVIDUAL ACTIVITIES=PERCENTILES

THE FOLLOWING DATA INCLUDE PRE-HATCH CHICKENS

11 DAY RAW DATA

011 2 1 0525 0800 0200
011 2 2
011 2 3 0660 0780 0220
011 2 4
011 2 5 1950 0710 0290
011 2 6
011 2 7 0495 0820 0180
011 2 8

011 2 1 0495 0647 0353
011 2 2
011 2 3 0822 0685 0315
011 2 4
011 2 5 1900 0566 0352 0082
011 2 6
011 2 7 0480 0533 0467
011 2 8

011 1 1 0525 0630 0370
011 1 2
011 1 3 0855 0662 0338
011 1 4
011 1 5 1920 0633 0367
011 1 6
011 1 7 0285 0630 0370
011 1 8

011 1 1 0555 0571 0429
011 1 2

011 1 3 0692 0632 0368
011 1 4
011 1 5 1970 0569 0371 0060
011 1 6
011 1 7 0300 0611 0389
011 1 8

11-DAY MEANS

011 1 0525 0662 0338
011 2
011 3 0757 0689 0310
011 4
011 5 1935 0619 0345 0035
011 6
011 7 0390 0648 0351
011 8

15 DAY RAW DATA

015 2 1 0390 0471 0456 0073
015 2 2
015 2 3 1470 0586 0345 0043 0026
015 2 4
015 2 5 1650 0605 0355 0040
015 2 6
015 2 7 0330 0560 0440
015 2 8

015 2 1 0330 0581 0387 0032
015 2 2
015 2 3 1360 0600 0333 0067
015 2 4
015 2 5 1687 0661 0286 0054
015 2 6
015 2 7 0413 0563 0406 0031
015 2 8

015 1 1 0240 0439 0439 0122
015 1 2
015 1 3 1110 0496 0366 0092 0046
015 1 4
015 1 5 2025 0712 0288
015 1 6
015 1 7 0705 0495 0400 0105
015 1 8

015 1 1 0300 0553 0395 0053
015 1 2
015 1 3 1220 0633 0347 0020
015 1 4
015 1 5 1987 0610 0341 0049
015 1 6
015 1 7 0623 0595 0405
015 1 8

15-DAY MEANS

015 1 0315 0511 0419 0070

015	2					
015	3	1290	0578	0347	0055	0036
015	4					
015	5	1837	0647	0317	0035	
015	6					
015	7	0518	0553	0412	0034	
015	8					

17 DAY RAW DATA

017	2	1	0730	0491	0421	0088
017	2	2				
017	2	3	1060	0592	0340	0068
017	2	4				
017	2	5	1470	0514	0297	0090 0099
017	2	6				
017	2	7	0600	0375	0500	0125
017	2	8				
017	2	1	0525	0469	0469	0062
017	2	2	0278	0295	0591	0114
017	2	3	1365	0557	0293	0086 0064
017	2	4				
017	2	5	1410	0718	0282	
017	2	6	0315	0257	0314	0157 0271
017	2	7	0615	0517	0400	0083
017	2	8				
017	1	1	0530	0595	0351	0254
017	1	2				
017	1	3	1370	0613	0339	0048
017	1	4				
017	1	5	1260	0509	0284	0103 0103
017	1	6				
017	1	7	0960	0354	0481	0165
017	1	8				
017	1	1	0735	0451	0451	0098
017	1	2	0300	0297	0595	0108
017	1	3	1065	0672	0295	0033
017	1	4				
017	1	5	1320	0484	0336	0090 0090
017	1	6	0225	0246	0316	0180 0259
017	1	7	0945	0310	0394	0141 0155
017	1	8				

17-DAY MEANS

017	1	0630	0501	0423	0075	
017	2	0289	0296	0593	0111	
017	3	1215	0608	0316	0058	0032
017	4					
017	5	1365	0556	0299	0070	0073
017	6	0270	0251	0315	0435	
017	7	0780	0389	0443	0127	0038
017	8					

19 DAY RAW DATA

019	2	1	0450	0538	0338	0124
019	2	2	0285	0500	0500	
019	2	3	1260	0614	0325	0060
019	2	4				
019	2	5	1500	0509	0327	0145 0018
019	2	6	0450	0700	0300	
019	2	7	0300	0435	0478	0087
019	2	8	0330	0459	0405	0135
019	2	1	0630	0603	0365	0032
019	2	2	0345	0480	0530	
019	2	3	1170	0708	0270	0022
019	2	4				
019	2	5	1500	0589	0271	0065 0065
019	2	6	0690	0581	0387	0032
019	2	7	0510	0482	0411	0107
019	2	8	0405	0401	0592	
019	1	1	0300	0587	0370	0043
019	1	2	0435	0450	0550	
019	1	3	1410	0754	0246	
019	1	4				
019	1	5	1530	0549	0329	0073 0049
019	1	6	0585	0743	0257	
019	1	7	0570	0324	0397	0118 0162
019	1	8	0360	0524	0476	
019	1	1	0450	0511	0420	0068
019	1	2	0195	0470	0530	
019	1	3	1095	0647	0353	
019	1	4				
019	1	5	1545	0483	0325	0083 0108
019	1	6	0450	0450	0500	0050
019	1	7	0300	0393	0508	0098
019	1	8	0375	0465	0535	

19-DAY MEANS

019		1	0457	0559	0373	0067
019		2	0318	0475	0525	
019		3	1233	0680	0298	0020
019		4				
019		5	1518	0532	0313	0091 0060
019		6	0543	0619	0361	0020
019		7	0420	0408	0448	0102 0041
019		8	0367	0433	0567	

THE FOLLOWING DATA INCLUDE POST-HATCH CHICKENS

DAY 1 RAW DATA

001	2	1	0600	0522	0391	0087	
001	2	2	0570	0543	0343	0114	
001	2	3	1725	0842	0158		
001	2	4	1785	0492	0322	0051	0068 0068
001	2	5	1665	0429	0341	0088	0088 0055

001 2 6 0420 0317 0415 0122 0098 0049
001 2 7 0855 0206 0278 0299 0216
001 2 8 0480 0623 0377

011 1 1 0375 0506 0420 0165
001 1 2 0780 0394 0455 0152
001 1 3 1350 0753 0224 0124
001 1 4 1350 0545 0327 0129
001 1 5 1515 0453 0324 0169 0054
001 1 6 0450 0478 0400 0077 0046
001 1 7 0600 0339 0312 0349
001 1 8 0450 0687 0313

1-DAY MEANS

001 1 0487 0514 0140 0076
001 2 0675 0468 0399 0133
001 3 1537 0797 0197 0012
001 4 1567 0518 0325 0090 0034 0034
001 5 1590 0441 0333 0128 0071 0028
001 6 0421 0397 0407 0099 0072 0024
001 7 0725 0272 0295 0324 0108
001 8 0465 0645 0355

DAY 7 RAW DATA

007 2 1 0780 0655 0345
007 2 2 0450 0542 0458
007 2 3 1410 0860 0088 0052
007 2 4 0960 0623 0340 0038
007 2 5 1050 0423 0356 0158 0132
007 2 6 0675 0342 0368 0158 0132
007 2 7 0795 0358 0309 0160 0173
007 2 8 0570 0683 0269 0048

007 2 1 0476 0625 0333 0041
007 2 2 0585 0474 0526
007 2 3 1185 0760 0206 0032
007 2 4 1080 0518 0311 0094 0075
007 2 5 1440 0474 0171 0232 0090 0030
007 2 6 0725 0353 0380 0140 0126
007 2 7 0685 0340 0297 0159 0138 0064
007 2 8 0690 0434 0368 0197

007 1 1 0795 0789 0211
007 1 2 0600 0600 0400
007 1 3 1290 0918 0082
007 1 4 1005 0559 0356
007 1 5 1170 0382 0382 0092 0118 0026
007 1 6 0840 0326 0384 0163 0128
007 1 7 0585 0357 0387 0153 0102
007 1 8 0870 0563 0375 0062

007 1 1 0650 0614 0368 0018
007 1 2 0600 0571 0429
007 1 3 1110 0897 0102
007 1 4 0900 0544 0316 0069 0069
007 1 5 0930 0349 0388 0116 0145
007 1 6 0525 0392 0388 0100 0120

007 1 7 0720 0250 0355 0157 0236
007 1 8 0675 0482 0448 0068

7-DAY MEANS

007 1 0675 0670 0314 0015
007 2 0557 0474 0436 0090
007 3 1247 0839 0147 0013
007 4 0986 0563 0330 0063 0044
007 5 1147 0402 0369 0125 0125 0014
007 6 0691 0353 0380 0140 0126
007 7 0696 0326 0337 0157 0162 0016
007 8 0701 0540 0365 0093

DAY 14 RAW DATA

014 2 1 0450 0577 0371 0052
014 2 2 0375 0500 0455 0045
014 2 3 1065 0755 0186 0059
014 2 4 0555 0615 0346 0019 0019
014 2 5 1560 0412 0321 0134 0116 0018
014 2 6 0570 0350 0313 0175 0138 0025
014 2 7 0900 0198 0228 0228 0139 0208
014 2 8 0450 0679 0321

014 1 1 0600 0583 0369 0048
014 1 2 0645 0435 0464 0101
014 1 3 1605 0833 0091 0030 0045
014 1 4 0750 0573 0360 0027 0040
014 1 5 0930 0447 0379 0097 0068 0010
014 1 6 0675 0349 0373 0145 0133
014 1 7 0705 0355 0318 0173 0154
014 1 8 0750 0612 0388

14-DAY MEANS

014 1 0525 0581 0370 0050
014 2 0533 0449 0456 0092
014 3 1337 0794 0138 0045 0022
014 4 0653 0594 0353 0023 0030
014 5 1245 0430 0350 0115 0092 0014
014 6 0623 0350 0343 0160 0135 0130
014 7 0803 0276 0273 0200 0146 0104
014 8 0600 0645 0355

DAY 30 RAW DATA

030 2 1 0675 0649 0297 0054
030 2 2 0780 0568 0389 0042
030 2 3 1290 0854 0146
030 2 4 1005 0551 0308 0065 0047 0028
030 2 5 1335 0457 0349 0128 0056
030 2 6 1320 0392 0378 0216 0013
030 2 7 1140 0257 0243 0203 0297
030 2 8 0615 0589 0300 0311

030 1 1 0795 0701 0299
030 1 2 0630 0356 0542 0102

030	1	3	1410	0885	0115		
030	1	4	1170	0595	0367	0038	
030	1	5	1050	0443	0333	0123	0101
030	1	6	0990	0362	0340	0160	0128	0011
030	1	7	0900	0228	0299	0134	0150	0189
030	1	8	0690	0447	0447	0106		

30-DAY MEANS

030	1	0735	0675	0298	0027		
030	2	0705	0462	0465	0073		
030	3	1350	0869	0131			
030	4	1087	0573	0337	0051	0024	0014
030	5	1192	0450	0341	0125	0079	
030	6	1155	0377	0359	0188	0071	
030	7	1020	0242	0271	0168	0223	0095
030	8	0652	0518	0373	0109		

DAY 60 RAW DATA

060	2	1	0900	0597	0403		
060	2	2	0675	0532	0437	0032	
060	2	3	1710	0819	0181		
060	2	4	0885	0511	0330	0106	0053
060	2	5	1575	0408	0308	0154	0131
060	2	6	0945	0397	0397	0132	0074
060	2	7	0460	0022	0370	0152	0196	0261
060	2	8	0660	0652	0319	0029		

060	2	1	0555	0552	0418	0030		
060	2	2	0600	0453	0467	0080		
060	2	3	1470	0797	0165	0038		
060	2	4	0615	0529	0189	0119	0137	0048
060	2	5	1365	0411	0286	0119	0137	0048
060	2	6	0825	0350	0360	0140	0090	0060
060	2	7	0570	0033	0131	0246	0295	0295
060	2	8	0570	0605	0368	0026		

060	1	1	0810	0609	0261	0130		
060	1	2	0600	0500	0357	0143		
060	1	3	1650	0906	0094			
060	1	4	0810	0514	0324	0135	0027	
060	1	5	1260	0426	0278	0074	0130	0074
060	1	6	1050	0313	0344	0094	0125	0125
060	1	7	0495	0114	0224	0211	0329	0237
060	1	8	0675	0556	0378	0067		

060	1	1	0705	0614	0386		
060	1	2	0735	0438	0453	0109	
060	1	3	2250	0762	0190	0048	
060	1	4	1155	0496	0389	0088	0026	
060	1	5	1695	0374	0367	0136	0109	0014
060	1	6	1095	0422	0313	0145	0096	0024
060	1	7	0570	0035	0232	0268	0339	0155
060	1	8	0525	0592	0300	0099		

60-DAY MEANS

060	1	0742	0593	0367	0040		

060	2	0653	0480	0428	0192		
060	3	1770	0821	0157	0021		
060	4	0866	0512	0333	0112	0060	0012
060	5	1473	0405	0309	0096	0126	0039
060	6	0960	0370	0353	0127	0096	0052
060	7	0524	0052	0239	0219	0289	0237
060	8	0607	0601	0341	0055		

DAY 120 RAW DATA

120	2	1	0595	0613	0386		
120	2	2	0390	0633	0367		
120	2	3	1400	0909	0091		
120	2	4	1045	0651	0325	0023	
120	2	5	1480	0343	0386	0128	0141
120	2	6	0645	0375	0367	0164	0093
120	2	7	0645	0000	0211	0145	0303	0342
120	2	8	0600	0507	0462	0029		
120	2	1	0765	0667	0333		
120	2	2	0420	0744	0256		
120	2	3	1515	0807	0193		
120	2	4	1080	0627	0254	0051	0051	0017
120	2	5	1350	0332	0332	0123	0123	0088
120	2	6	0600	0306	0388	0120	0157	0028
120	2	7	0705	0000	0259	0070	0253	0418
120	2	8	0555	0511	0422	0067		
120	1	1	0555	0742	0256		
120	1	2	0210	0667	0333		
120	1	3	1320	0940	0060		
120	1	4	0975	0528	0340	0034	0034	0052
120	1	5	1530	0282	0333	0154	0179	0051
120	1	6	0735	0383	0280	0167	0117	0050
120	1	7	0625	0000	0237	0105	0267	0391
120	1	8	0705	0567	0358	0075		
120	1	1	0725	0659	0341		
120	1	2	0240	0811	0189		
120	1	3	1435	0823	0177		
120	1	4	1020	0426	0360	0126	0080	0010
120	1	5	1400	0376	0304	0134	0116	0067
120	1	6	0695	0423	0343	0124	0080	0029
120	1	7	0685	0000	0233	0109	0289	0369
120	1	8	0660	0539	0390	0071		

120-DAY MEANS

120	1	0660	0670	0329			
120	2	0315	0713	0287			
120	3	1417	0869	0131			
120	4	1033	0558	0319	0058	0041	0020
120	5	1440	0333	0338	0134	0139	0051
120	6	0670	0371	0345	0143	0111	0026
120	7	0665	0000	0235	0107	0278	0380
120	8	0630	0546	0398	0053		

ADULT RAW DATA

00A	2	1	0555	0629	0241	0129	
00A	2	2					
00A	2	3	1920	0856	0144		
00A	2	4	0885	0626	0230	0131	0010
00A	2	5	1470	0379	0270	0218	0091
00A	2	6	0690	0393	0323	0182	0142	0051
00A	2	7	0990					1000
00A	2	8	0675	0521	0420	0057		
00A	2	1	0530	0547	0410	0041		
00A	2	2						
00A	2	3	1845	0783	0217			
00A	2	4	0925	0443	0272	0113	0170	
00A	2	5	1425	0326	0300	0153	0146	0073
00A	2	6	0845	0379	0310	0180	0126	0022
00A	2	7	0880					1000
00A	2	8	0795	0478	0347	0173		
00A	1	1	0570	0613	0329	0056		
00A	1	2						
00A	1	3	1680	0753	0188	0054		
00A	1	4	0900	0412	0317	0111	0111	0047
00A	1	5	1350	0264	0205	0132	0176	0220
00A	1	6	1080	0386	0295	0136	0113	0068
00A	1	7	0690					1000
00A	1	8	0975	0571	0428			
00A	1	1	0590	0663	0336			
00A	1	2						
00A	1	3	1755	0866	0133			
00A	1	4	0860	0600	0286	0066	0047	
00A	1	5	1395	0347	0305	0203	0143	
00A	1	6	0945	0382	0234	0278	0104	
00A	1	7	0800					1000
00A	1	8	0855	0571	0535	0096		

ADULT MEANS

00A		1	0560	0613	0329	0056		
00A		2						
00A		3	1800	0814	0170	0013		
00A		4	0892	0520	0276	0105	0084	0012
00A		5	1410	0329	0270	0176	0139	0073
00A		6	0895	0385	0267	0191	0121	0025
00A		7	0840					1000
00A		8	0825	0535	0387	0081		

ANNUITY MEANS